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(54) Title: CHIMERIC MOLECULES CONTAINING A MODULE ABLE TO TARGET SPECIFIC CELLS AND A MODULE REGULATING THE APOPTOGENIC FUNCTION OF THE PERMEABILITY TRANSITION PORE COMPLEX (PTPC)

(57) Abstract: A chimeric polypeptide has the formula: pTox-pTarg, wherein pTox is a viral apoptotic peptide, such as the Vpr peptide of HIV-1 or a fragment of the Vpr peptide of HIV-1 containing the amino acid motif H(F/S)RIG that interacts with mitochondrial inner membrane, adenine nucleotide translocation (ANT) protein of a cell. pTarg is an antibody or an antibody fragment that binds to the outer membrane of the cell. Binding of the chimeric polypeptide to the cell is followed by apoptosis of the cell. A vector encoding a chimeric polypeptide and a recombinant host cell comprising the vector are provided. The chimeric polypeptide us useful for targeting pTox to cells, such as cancer cells.

CHIMERIC MOLECULES CONTAINING A MODULE ABLE TO TARGET

SPECIFIC CELLS AND A MODULE REGULATING THE APOPTOGENIC

FUNCTION OF THE PERMEABILITY TRANSITION PORE COMPLEX (PTPC)

CROSS-REFERENCE TO RELATED APPLICATIONS

The application hereby claims the benefit under 35 U.S.C. § 119(e) of United States provisional application Serial No. 60/265,594, filed February 2, 2001. The entire disclosure of this application is relied upon and incorporated by reference herein.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to cell death regulatory molecules for therapeutic use. More specifically, this invention relates to molecules in which a peptidic or pseudo-peptidic part acting on the permeability transition pore complex (PTPC) is covalently linked to cell-targeting molecules including antibodies, recombinant antibody fragments or homing peptides. The resulting chimeric molecules are polypeptides or peptidomimetic molecules which target the PTPC and/or its major component the adenine nucleotide translocation (ANT) to induce or inhibit cell death (apoptosis). This invention also relates to such chimeric molecules when the PTPC-interacting part is an apoptogenic HIV-1 Vpr-derived peptide (or pseudo-peptide) or an ANT-derived peptide (or pseudo-peptide). This invention also relates to nucleic acid sequence construct encoding such chimeric molecule or encoding portions of these chimeric molecules.

Background

It is currently agreed that mitochondria play an important role in controlling life and death of cells (apoptosis; Kroemer and Reed 2000, Nature Medicine). It appears both that an increasing number of molecules involved in the transduction of the signal and also many metabolites and certain viral effectors act on mitochondria and influence the permeabilisation of mitochondrial membranes. Using mitochondrial-specific pro-apoptotic agent would seem to be an emerging concept in cancer therapy (Costantini et al 2000, Journal of the National Cancer Institute). Similarly, it might be possible to use cytoprotective molecules, thanks to their ability to stabilize mitochondrial membranes, in the treatment of illnesses where there is excessive apoptosis (neurodegenerative diseases, ischemia, AIDS, fulminant hepatitis, etc.).

Mitochondrial membrane permeabilisation (MMP) is a key event of apoptotic cell death associated with the release of caspase activators and caspase-independent death effectors from the intermembrane space, dissipation of the inner transmembrane potential (ΔΨm), as well as a perturbation of oxidative phosphorylation (Green and Reed, 1998; Gross *et al.*, 1999; Kroemer and Reed, 2000; Kroemer *et al.*, 1997; Lemasters et al., 1998; Vander Heiden and Thompson, 1999; Wallace, 1999). Pro- and anti-apoptotic members of the Bcl-2 family regulate inner and outer MMP through interactions with the adenine nucleotide translocation (ANT; in the inner membrane, IM), the voltage-dependent anion channel (VDAC; in the outer membrane, OM), and/or through autonomous channel-forming activities (Desagher et *al.*, 1999; Gross et al., 1999; Kroemer and Reed, 2000; Marzo et al., 1998; Shimizu et al., 1999; Vander Heiden and Thompson, 1999). ANT and VDAC are major components of the permeability transition pore complex (PTPC), a polyprotein structure organized at sites at which the two mitochondrial membranes are apposed (Crompton, 1999; Kroemer and Reed, 2000).

The mitochondrial phase is under the control of Bcl-2 family of oncogenes and anti-oncogenes (for review: 5; 28) involved in more than 50% of cancers (29). All members of Bcl-2 family play an active role in the regulation of apoptosis, some of them being proapoptotic (Bax, Bak, Bcl- X_S, Bad, etc.) and others, being antiapoptotic (Bcl-2, Bcl-X_L, Bcl-w, Mcl-l, etc.) (G. Kroemer, Nat Med 3, 614-20 (1997)).

The mitochondrial megachannel is a polyprotein complex formed in the contact site between the inner and the outer mitochondrial membranes that participate in the regulation of mitochondrial membrane permeability. It is composed of a set of proteins including mitochondrion-associated hexokinase (HK), porin (voltage-dependent anion channel or VDAC), adenine nucleotide translocation (ANT), peripheral benzodiazepin receptor (PBR), creatine kinase (CK), and cyclophilin D, as well as Bcl-2 family members. In physiological conditions, PTPC controls the mitochondrial calcium homeostasis via the regulation of its conductance by the mitochondrial pH, the ΔΨm, NAD/NAD(P)H redox equilibrium and matrix protein thiol oxidation. (M. Zoratti, I. Szabo, Biochim, Biophys Acta 1241, 139-76 (1995). S. Shimizu, M. Narita, Y. Tsujimoto, Nature 399, 483-487 (1999). M. Crompton, Biochem J 341, 233-249 (1999). K. Woodfield, A. Ruck, D. Brdiczka, A. P. Halestrap, Biochem J 336, 287-90 (1998).

P. Bernardi, K. M. Broekemeier, D. R. Pfeiffer, J Bioenerg Biomembr 26, 509-17 (1994). F. Ichas, L. Jouaville, J. Mazat, Cell 89, 1145-53 (1997)).

Apoptosis and related forms of controlled cell death are involved in a great number of illness. Excess or insufficiency of cell death processes are involved in auto-immune and neurodegenerative diseases, cancers, ischemia, and pathological infections or diseases such as viral and bacterial infections. Just few examples illustrating the virtually ubiquitous involvement of mitochondria in diseases associated with the abnormal control of cell death will be mentioned here.

In different models of ischemia (heart, liver, kidney or brain), using molecules that are capable of stabilising mitochondrial membranes, such as CsA for example (or its analogous non-immunosuppressor –Me-Val4-CsA) has made it possible to reduce massive apoptosis and its acute consequences at the level of the organ. In addition, VDAC is indispensable for the destruction of neurons of the rat hippocampus after hypoxic reperfusion. In the area of neurodegenerative diseases, a great many observations suggest close links with mitochondrial control of apoptosis (see Kroemer and Reed 2000, Nature Medicine). The neurotoxin –methyl-4-phenylpyridinium induces mitochondrial permeability transition and the exit of cytochrome c. Poisoning by mitochondrial toxins such as nitro-propionic acid or rotenone provokes in primates and rodents a Huntington-disease type of illness.

PTPC is a dynamic protein complex located at the contact site between the two mitochondrial membranes, its opening allowing the free diffusion of solutes < 1500 Da on the inner membrane. Formation of PTPC involves the association of proteins from different compartments, hexokinase (cytosol), porin, also called voltage-dependent anion channel (VDAC, outer membrane), peripheral benzodiazepin receptor (PBR, outer membrane), ANT (inner membrane) and cyclophilin D (matrix). PTPC has been implicated in many examples of apoptosis due to its capacity to integrate multiple pro-apoptotic signal transduction pathways and due to its control by proteins from Bcl-2/Bax family. The Bcl-2 family comprises death inhibitory (Bcl-2-like) and death inducing (Bax-like) members which respectively prevent or facilitate PTPC opening. Bax and Bcl-2 reportedly interact with VDAC and ANT within PTPC. In physiological conditions, ANT is a specific antiporter for ADP and ATP. However, ANT can also form a lethal pore upon interaction with different pro-apoptotic agents. including Ca2+,

atractyloside, HIV-1 Vpr-derived peptides and pro-oxidants. Mitochondrial membrane permeabilization may also be regulated by the non-specific VDAC pore modulated by Bcl-2/Bax-like proteins in the outer membrane (12; 16). and/or by changes in the metabolic ATP/ADP gradient between the mitochondrial matrix and the cytoplasm (17).

There is a need in the art for cytoprotective molecules in ischemia, neurodegenerative diseases, fulminant hepatitis and viral infections.

Another application of the chimeric molecule according the invention can be contemplated for the preparation of cosmetics or for preventing early death of plants or vegetables or flowers particularly for preventing the opening of the PTPC.

Conventional chemotherapeutic agents are limited in their therapeutic effectiveness by severe side effects due to their poor selectivity for tumors. The development of monoclonal antibodies (and ScFv) against specific tumor antigens and the identification of homing peptides specific for tumor vascularisation have made it possible to consider enhancing the selectivity of anticancer drugs by a targeted delivery approach. However, such reported attempts using monoclonal antibodies and the anticancer drugs doxorubicin (Trail P.A., et al 1993 Science 261:212), metotrexate (Kanellos J. et al., 1985 J Natl Cancer Inst 75:319), and Vinca alkaloids (Starling J.J. et al., 1991 Cancer Res 41:2965), have been largely unsuccessful. These antibodydrug conjugates were only moderately potent and usually less cytotoxic than the corresponding unconjugated drugs. In fact, antigen-specific cytotoxicity toward cultured tumor cells was rarely demonstrated. In vivo therapeutic effects with these conjugates in tumor zenograft animal models were in general observed only when the treatments were commenced before the tumors were well established or when exceedingly large doses (up to 90 mg/kg, drug equivalent dse) were used. It is, therefore, not surprising that in human clinical trials, no significant antitumor effects were observed with these agents (Elias D.J. et al., 1994 Am Respir Crit Care Med 150:1114) (Schneck D. et al., 1990). Indeed, the peak circulating serum concentrations of conjugates were only in the same range as their in vitro IC50 value and thus, capable of eliminating at best only about 50% of tumor cells.

These observations led to the conclusion that the previous attempts at delivering therapeutic doses of cytotoxic drugs via monoclonal antibodies have met with little success in clinical trials because of inappropriate choice of drug. One possible (partial-) solution was to

conclude that immunoconjugates must be composed of drugs possessing much higher potency than the clinically used anticancer agents if therapeutic levels of conjugate at the tumor sites are to be achieved in patients. Effectively, such toxins, including maytansinoides, enedignes, or intercalating agents CC1065, were shown to be 100 to 1000-fold more cyctotoxic than the chemotherapeutic agents doxorubicin, methotrexate, and Vinca alkaloids (Chari RVJ et al., 1995 Cancer Res 55:4079) (Chari RVJ et al., 1992, Cancer Res 52:127).

Another approach termed "Adept" was also designed. This antibody-directed enzyme prodrug therapy (Adept) is based upon the use of a monoclonal antibody to target an enzyme at the tumor cell surface, which ultimately is expected to selectively deliver an antitumor drug from a suitable inactive prodrug. In both cases, clinical trials are in progress; however, since today none of them have been introduced in cancer chemotherapy, there is a need for new tools to kill target tumor cells. Bagshawe KD, 1990. Antibody-directed enzyme/prodrug therapy (ADEPT). Biochem Soc Trans. 18(5):750-2. Melton RG, Sherwood RF. 1996 Antibody-enzyme conjugates for cancer therapy. J Natl Cancer Inst, 88(3-4):153-65. Rihova B. 1997; Targeting of drugs to cell surface receptors. Crit Rev Biotechnol. 17(2):149-69. Hudson PJ. 2000. Recombinant antibodies: a novel approach to cancer diagnosis and therapy. Expert Opin Investig Drugs 9(6):1231-42.

Recently, the mitochondrion has been proposed as a novel prospective target for chemotherapy-induced apoptosis (1-7). Indeed, four different anti-cancer agents, including the resinoid acid-derivative CD437, lonidamine, betulinic acid, and arsenite, have been shown to induce cancer cell apoptosis by a direct action on mitochondria. The interaction of these anti-cancer agents with mitochondria results in an increase of the permeability of the inner mitochondrial membrane due, at least in part, to the opening of the permeability transition pore complex (PTPC). PTPC opening leads to swelling of the mitochondria matrix, the dissipation of the inner transmembrane potential ($\Delta\Psi$ m), enhanced generation of reactive oxygen species (ROS), and the release of apoptogenic proteins from the intermembrane space to the cytoplasm. Such mitochondrial apoptogenic effectors include the caspase activator cytochrome c, apoptosis inducing factor (AIF), and pro-caspases (2-6). All the signs of apoptosis induced by CD437, lonidamine, betulinic acid, and arsenite are prevented by two agents acting on specific PTPC proteins, namely cyclopsporin A (CsA, a cyclophilin D ligand) and bongkrekic acid (BA, a

ligand of the adenine nucleotide translocase (ANT)). It thus appears that PTPC opening is a critical event of apoptosis triggered by these agents.

Mastoparan, a peptide isolated from wasp venom, is the first peptide known to induce mitochondrial membrane permeabilization via a CsA-inhibitable mechanism and to induce apoptosis via a mitochondrial effect when added to intact cells. This peptide has an α -helical structure and possesses some positive charges that are distributed on one side of the helix. A similar peptide (KLAKLAKLAKLAK or (KLAKLAK)₂ (K = lysine, L = alanine, and A = leucine) has been found recently to disrupt mitochondrial membranes when it is added to purified mitochondria, although the mechanisms of this effect have not been elucidated.

The vasculature of individual tissues is highly specialized. The endothelium in lymphoid tissues expresses tissue-specific receptors for lymphocyte homing, and recent work utilizing phage homing has revealed an unprecedented degree of specialization in the vasculature of other normal tissues. *In vivo* screening of libraries of phage that displace random peptide sequences on their surfaces has yielded specific homing peptides for a large number of normal tissues. The tissue-specific endothelial molecules to which the phage peptides home may serve as receptors for metastasizing malignant cells. Probing of tumor vasculature has yielded peptides that home to endothelial receptors expressed selectively in angiogenic neovasculature. These receptors, and those specific for the vasculature of individual normal tissues, are likely to be useful in targeting therapies to specific sites. Ruoslahti E, Rajotte D. 2000; An address system in the vasculature of normal tissues and tumors. Annu Rev Immunol. 18:813-27.

Ellerby et al. recently have fused the mitochondriotoxic (KLAKLAK)₂ motif to a targeting peptide that interacts with endothelial cells. Such a fusion peptide is internalized and induces mitochondrial membrane permeabilization in angiogenicendothelial cells and kills MDA-MD-435 breast cancer xenografts transplanted into nude mice. Similarly, a recombinant chimeric protein containing interleukin 2 (IL-2) protein fused to Bax selectively binds to and kills IL-2 receptor-bearing cells *in vitro*. Thus, specific cytotoxic agents that target surface receptors, translocate into the cytoplasm, and induce apoptosis via mitochondrial membrane permeabilization might be useful in treating cancer.

There is a need in the art for the selective eradication of transformed cells. One strategy is to target a toxic agent to selected cell types. More particularly, there exists a need in the art for method and reagents for regulating mitochondrial permeabilization and apoptosis.

Summary of the Invention

In order to overcome at least some of the limitations of the prior art, the present invention provides a peptidic or pseudo-peptidic family of polyfunctional molecules containing a cell-targeting part (termed TARG), a PTPC-interacting part (termed TOX/SAVE), and a facultative mitochondrial localisation sequence (MLS). In a preferred embodiment of the invention, the TOX/SAVE portion of the said polyfunctional molecule is a peptide or peptidomimetic molecule which interact directly with the Adenine Nucleotide Translocator (ANT) a central component of the PTPC

Thus, the present invention includes two categories of targeted cell death regulatory molecules:

- TARG-(MLS)-TOX is a polyfunctional molecule which induces a PTPC-dependent mitochondrial membrane permeabilisation and consequent cell death.
- TARG-(MLS)-SAVE is a polyfunctional molecule which protects cells from mitochondrial membrane permeabilisation and consequently from cell death through interaction with the PTPC and/or ANT.

The invention further provides a vector encoding a chimeric polypeptide of the invention. Also, the invention provides a recombinant host cell comprising a vector of the invention.

Further, the invention provides a cancer cell having a tumor-associated antigen on the surface thereof to which the chimeric polypeptide of the invention is bound via the antibody or antibody fragment of the chimeric polypeptide. The invention also provides methods for detecting cancer cells.

The invention also provides methods for inducing or preventing apoptosis with polypeptides of the invention. The invention provides methods for inducing apoptosis in tumor cells. The invention provides methods for inducing apoptosis in virus infected cells.

The invention further provides hybridomas producing polypeptides of the invention. The invention also provides monoclonal antibodies produced by these hybridomas.

The invention also provides methods for identifying active agents of interest that interact with the PTPC. The invention also provides methods for identifying active agents of interest that interact with ANT peptide. The invention also provides methods for identifying mitochondrial antigens.

The invention also provides methods of treatment or prevention of a pathological infection or disease by administering a polypeptide of the invention to a patient. The invention also provides pharmaceutical compositions comprising a polypeptide of the invention.

Brief Description of the Drawings

- Figure 1 shows the nucleotide sequence of vector pACgp67-ScFv461.
- Figure 2 shows the nucleotide sequence of vector pACgp67-ScFv350.
- Figure 3 shows the nucleotide sequence of Vh and VL, from the clone therap 99B3.
- Figure 4 shows the nucleotide sequence of Vh and VL from the clone therap.88E10.
- Figure 5 shows the nucleotide sequence of Vh and VL from the clone therap.152C3.
- Figure 6, 7, 8, 9, 10, 11 show surface plasmon resonance curves.
- Figures 12 and 13 show the strategy for obtaining the ScFv-transfert vector.

Detailed Description of the Invention

It was recently discovered that the proapoptotic HIV-1-encoded protein Vpr induces mitochondrial membrane permeabilization via its physical and functional interaction with the mitochondrial inner membrane protein ANT (adenine nucleotide translocation, also called ADP/ATP carrier). This was shown using a variety of different techniques: surface plasmon resonance, electrophysiology, synthetic proteoliposomes, studies on purified mitochondria (respirometry, electron microscopy, organellofluorometry), as well as microinjection of intact cells. These discoveries are described in detail in U.S. Provisional Application No. 60/231,539 filed September 11, 2000, the entire disclosure of which is relied upon and incorporated by reference herein.

The present invention pertains to novel cytotoxic conjugates based on the association between a peptidic molecule (named pTox) interacting with the mitochondrial permeability transition pore complex (PTPC) and a molecule (named pTarg) able to target cells. The present

invention also pertains to novel cytoprotective conjugates based on the association between a peptidic molecule (named SAVE) interacting with the mitochondrial permeability transition pore complex (PTPC) and a molecule (named pTarg) able to target the cells to rescue. In a specific embodiment of this invention, a cytotoxic conjugate of the invention includes a viral derived proapoptotic peptide.

In one embodiment of the invention, the polyfunctional molecule TARG-(MLS)-TOX is a tumor specific molecule that selectively interact with a tumor cell or a specific mammalian cell type, where the polyfunctional molecule is selectively internalised by the mammalian or tumoral cell type, where the polyfunctional molecule interact with the PTPC and/or ANT and exhibits thereto a strong mitochondrio-toxicity leading to apoptosis or any cell death process.

In one embodiment of the invention, the polyfunctional molecule TARG-(MLS)-TOX exhibits a selective toxicity against angiogenic endothelial cells. In another embodiment of the invention, the polyfunctional molecule TARG-(MLS)-TOX exhibits a selective toxicity against tumor cells.

In one embodiment of the invention, the TARG part of the polyfunctional molecule TARG-(MLS)-TOX is an antibody or a recombinant antibody fragment. In another embodiment of the invention, the TARG part of the polyfunctional molecule TARG-(MLS)-TOX is tumor horning peptide (example; CNGRC peptide; lung-homing peptide CGFECVRQCPERC).

In one embodiment of the invention, the TOX part of the polyfunctional molecule TARG-(MLS)-TOX is a peptide or a peptido-mimetic derived from the C-terminal part (amino-acids 52 to 96) of the HIV-1 Vpr protein.

In one embodiment of the invention, the TOX part of the polyfunctional molecule TARG-(MLS)-TOX is a pro-apoptotic Bcl-2 family member such as the Bax or Bid proteins, or a fragment thereof.

In one embodiment of the invention, the TOX part of the polyfunctional molecule TARG-(MLS)-TOX is a D-peptide, is a Ψ-peptide or a retro-inverso peptide chosen among the group of peptidic sequences described in table 1:

Table I:

| Name | TOX Peptidic Sequences | |
|----------|------------------------|--|
| Vpr71-82 | HFRIGCRHSRIG | |

| Vpr71-82[R73,77,80K] | HFKIGCKHSKIG |
|------------------------------------|---|
| Vpr71-96 | HFRIGCRHSRIGIIQQRRTRNGASKS |
| Vpr71-96[R73,77,80K] | HFKIGCKHSKIGIIQQRRTRNGASKS |
| Vpr52-96 | DTWTGVEALIRILQQLLFIHFRIGCRHSRIGIIQQRRTRNGASKS |
| Vpr52-96[R73,77,80K] | DTWTGVEALIRILQQLLFIHFKIGCKHSKIGIIQQRRTRNGASKS |
| Vpr52-96[L60,67A] | DTWTGVEAAIRILQQALFIHFRIGCRHSRIGIIQQRRTRNGASKS |
| Vpr52-82 | DTWTGVEALIRILQQLLFIHFRIGCRHSRIG |
| Vpr52-82[R73,77,80K] | DTWTGVEALIRILQQLLFIHFKIGCKHSKIG |
| Histatin5 | DSHARKRHHGYKRKFHEKHHSHRGY |
| Candida Albicans | |
| Mastoparan | INLKALAALAKKIL |
| Vespula Lewisii | |
| hNUR77(555-568) | LSRLLGKLPELRTL |
| hNTR(368-381) | ATLDALLAALRRIQ |
| neutrotrophin receptor | |
| Bid(84-100) | RNIARHLAQVGDSMRDR |
| Bax(57-72) | KKLSECLKRIGDELDS |
| Bax(72-87) | GQVGRQLAIIGDDINR |
| HBX(70-78) | ALRFTSARR |
| DCC(1376-1390) | KTHVKTASLGLAGKA |
| ANT ₁ (104-116) | DRHKQFWRYFAGN |
| ANT ₂ (104-116) | DKRTQFWRYFAGN |
| ANT ₃ (104-116) | DKHTQFWRYFAGN |
| ANT ₁ (104-116 [A114P] | DRHKQFWRYFPGN |
| ANT ₂ (104-116)[A114P] | DKRTQFWRYFPGN |
| ANT ₃ (104-116)[A114P] | DKHTQFWRYFPGN |
| ANT _{1,2,3} (117-134) | LASGGAAGATSLCFVYPL |
| ANT ₁ (104-134) | DRHKQFWRYFAGNLASGGAAGATSLCFVYPL |
| ANT ₂ (104-134) | DKRTQFWRYFAGNLASGGAAGATSLCFVYPL |
| ANT ₃ (104-134) | DKHTQFWRYFAGNLASGGAAGATSLCFVYPL |
| ANT ₁ (104-134)[A114P] | DRHKQFWRYFPGNLASGGAAGATSLCFVYPL |
| ANT ₂ (104-134 [A114P] | DKRTQFWRYFPGNLASGGAAGATSLCFVYPL |
| ANT ₃ (104-134) [A114P] | DKHTQFWRYFPGNLASGGAAGATSLCFVYPL |
| Vpr 52-96 [C76S] | DTWTGVEALIRILQQLLFIHFRIGSRHSRIGIIQQRRTRNGASKS |
| HTLV-lp13II | 19PSLRVWRLCARRLV32 |

| Bad103-127 | NLWAAQRYGRELRRMSDEFVDSFKK |
|------------|---------------------------|
| Bax52-76 | QDASTKKLSECLKRIGDELDSNMEL |

In one embodiment of the invention, the SAVE part of the polyfunctional molecule TARG-(MLS)-SAVE is a L-peptide, a D-peptide or a retro-inverso peptide chosen among the group of peptidic sequences described in table II:

| Name | SAVE Peptidic Sequences | |
|--------------------------------|---------------------------------|--|
| ANT ₁ (104-116) | DRHKQFWRYFAGN | |
| ANT ₂ (104-116) | DKRTQFWRYFAGN | |
| ANT ₃ (104-116) | DKHTQFWRYFAGN | |
| ANT _{1,2,3} (117-134) | LASGGAAGATSLCFVYPL | |
| ANT ₁ (104-134) | DRHKQFWRYFAGNLASGGAAGATSLCFVYPL | |
| ANT ₂ (104-134) | DKRTQFWRYFAGNLASGGAAGATSLCFVYPL | |
| ANT ₃ (104-134) | DKHTQFWRYFAGNLASGGAAGATSLCFVYPL | |

In one embodiment of the invention, the TARG part of the polyfunctional molecule TARG-(MIS)-SAVE is a L-peptide, a D-peptide or a retro-inverso peptide chosen among the group of peptidic sequences described in table III:

| ANTENNAPEDIA | RQIKITFQNRRMKTKK |
|---------------------------|------------------|
| third helix (residues 43- | |
| 58) | |
| HIV-1 Vpr 83-96 | IIQQRRTRNGASKS |
| transduction domain | |
| HIV-1 Tat48-59 | GRKKRRQRRRPP |
| transduction domain | |
| HIV-1 Tat49-57 | RKKRRQRRR |
| transduction domain | |
| pep-1 | KETWWETWWTEW |

In one embodiment of the invention, the Targ part of the polyfunctionnal molecule TARG-(MLS)-TOX is the decanoic acid CH₃(CH₂)₈CO-.

In one embodiment of the invention, the TARG part of the polyfunctional molecule TARG-(MLS)-TOX is an antibody, a recombinant antibody, a recombinant antibody fragment or a ScFv (single chain fragment variable).

In one embodiment of the invention, the TARG part of the polyfunctional molecule TARG-(MLS)-TOX is encoded by the following vector pACgp67-ScFv461 (figure 1).

In one embodiment of the invention, the TARG part of the polyfunctional molecule TARG-(MLS)-TOX is encoded by the following vector pACgp67-ScFv350 (figure 2).

In one embodiment of the invention, the TARG part of the polyfunctional molecule TARG-(MLS)-TOX is a tumor homing peptide as defined by Ellerby et al in PCT/US00/01602.

In one embodiment of the invention, the TARG part of the polyfunctional molecule TARG-(MLS)-TOX/SAVE is a brain or kidney homing peptide as defined by Pasqualini R, Ruoslahti (in Nature 1996 Mar 28;380(6572):364-6. Organ targeting in vivo using phage display peptide libraries).

In one embodiment of the invention, pTox is the Vpr peptide of HIV-1 or a fragment thereof. Protein R (Vpr) of human immunodeficiency virus type 1 (HIV-1) is a virion-associated viral gene product with an average length of 96 amino acids, and a molecular weight of approximately 15 kD. Vpr is a highly conserved viral protein among HIV, simian immunodeficiency viruses (SIV). See Yuqi Zhao and Robert T. Elder, "Yeast Perspectives on HIV-1 VPR," Frontiers in Bioscience 5, d905-916, December 1, 2000.

Vpr has been characterized as an oligomer, and is thought to be divided into three domains on the basis of its structural features: an amino-terminal, negatively charged region that is predicted to form an amphipathic α helix (amino acids 17 to 34); a central hydrophobic domain (amino acids 35 to 75); and a carboxy-terminal, positively charged domain (amino acids 80 to 96). Mutational analysis of Vpr suggests that the nuclear import, virion incorporation, and cell cycle arrest of Vpr are mediated by the distinct functional domains. A structural motif within an amino-terminal helix appears to be important for packaging of Vpr into virions and for maintaining the stability of the protein. A central hydrophobic region, especially the leucine-isoleucine (LR) domain, is reported to be involved in the nuclear localization of Vpr. The cell

cycle arrest function of Vpr was found to be largely located within a carboxy-terminal, positively charged region. *See* Tomoyuki Yamaguchi, Nobumoto Watanabe, Hiromitsu Nakauchi, and Atsushi Koito, "Human Immunodeficiency virus type 1 Vpr Modifies Cell Proliferation via Multiple Pathways," Microbiol, Immunol., 43(5), 437-447, 1999.

The amino acid sequence of human immunodeficiency virus type 1 viral protein R (Vpr) is shown below:

MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYE TYGDTWAGVEAIIRILQQLLFIHFRIGCRHSRIGVTRQRRARNGASRS.

Vpr and peptides containing conserved H(F/S)RIG repeat motifs can rapidly penetrate human CD4 cells, and cause mitochondrial dysfunction and death by apoptosis. More particularly, recombinant Vpr and C-terminal peptides of Vpr containing the conserved sequence HFRIGCRHSRIG can cause permeabilization of CD4⁺ T lymphocytes, a dramatic reduction of mitochondrial membrane potential, and finally cell death. Vpr and Vpr peptides containing the conserved sequence rapidly penetrate cells, co-localize with the DNA, and cause increased granularity and formation of dense apoptotic bodies. Vpr treated cells undergo apoptosis, and this was confirmed by demonstration of DNA fragmentation. *See* C. Arunagiri, I. Macreadie, D. Hewish and A. Azad, "A C-terminal domain of HIV-1 accessory protein Vpr is involved in penetration, mitochondrial dysfunction and apoptosis of human CD4⁺ lymphocytes," Apoptosis 1997; 2: 69-76.

Using a yeast model system, it has been confirmed that there is a cytocidal activity associated with the C-terminal portion of Vpr, particularly the sequence HFRIGCRHSRIG. Vpr and portions of Vpr containing the sequence HFRIGCRHSRIG can kill a range of mammalian cells including human lymphocytes. *See* I.G. Macreadie, A, Kirkpatrick, P.M. Strike, and A.A. Azad, "Cytocidal Activities of HIV-1 VPR and Sac1p peptides Bioassayed in Yeast," Protein and Peptide Letters, Vol. 4, No. 3, pp. 181-186, 1997.

The C-terminal moiety (Vpr52-96), within an α-helical motif of 12 amino acids (Vpr71-82), contain several critical arginine (R) residues (R73, R77, R80), which are strongly conserved among different pathogenic HIV-1 isolates. L.G. Macreadie, et al., Proc. Natl. Acad. Sci. USA 92, 2770-2774 (1995). I.G. Macreadie, et al., FEBS Lett. 410, 145-149 (1997). E. Jacotot, et al., J. Exp. Med. 191, 33-45 (2000). Thus, the pro-apoptotic portion (pTox) of the chimeric

polypeptide of the invention can contain, for example, the sequence HFRIGCRHSRIG (HIV-1 Vpr71-82), HFKIGCKHSKIG, Vpr 71-96, Vpr 52-96, or a pseudo peptidic variant such as D[HFRIGCRHSRIG].

Other variants of Vpr peptides can also be employed in this invention. Peptide fragments of Vpr encompassing a pair of H(F/S)RIG sequence motifs (residues 71-75 and 78-82 of HIV-1 Vpr) have been shown cause cell membrane permeabilization and death in yeast and mammalian cells. Peptide Vpr $^{59-86}$ (residues 59-86 of Vpr) forms an α -helix encompassing residues 60-77, with a kink in the vicinity of residue 62. It has been shown that the first of the repeated sequence motifs (HFRIG) participates in a well-defined α -helical domain, whereas the second (HSRIG) lay outside the helical domain and forms a reverse turn followed by a less ordered region. On the other hand, peptides Vpr71-82 and Vpr71-96, in which the sequence motifs are located at the N-terminus, were largely unstructured under similar conditions, as judged by their C²H chemical shifts. Thus, it has been shown that the HFRIG and HSRIG motifs adopt α-helical and turn structures, respectively, when preceded by a helical structure, but are largely unstructured in isolation. There are implications of these findings for interpretation of the structure-function relationships of synthetic peptides containing these motifs. For example, since the HFRIG and HSRIG sequence motifs adopt helical and turn structures, respectively, when preceded by a helical structure, as in full-length Vpr, but are largely unstructured in isolation, 7-8 residues, sufficient to support at least 1-2 turns of helix, should be included at the N-terminus of Vpr when used as the pTox component of the chimeric polypeptides of the invention to ensure that they are able to adopt the same structure as in the full-length protein. See Shenggen Yao, Allan M. Torres, Ahmed A. Azad, Ian G. Macreadie and Raymond S. Norton, "Solution Structure of Peptides from HIV-1 Vpr Protein that Cause Membrane Permeabilization and Growth Arrest," J. Peptide Sci. 4: 426-435 (1998). While the Vpr gene codes for a protein of 96-amino-acids, variations have been observed, e.g., Vprs from HIV-1_{HXB2} have 97 and 90-amino-acid residues, respectively. It will be understood that these variants can also be employed in this invention.

For the most effective toxicity, HFRIGCRHSRIG should be surrounded on each side by about eight amino acids from the native sequence. Vpr polypeptides and peptides of greater than 9 amino acids that inhibit or augment Vpr binding, mitochondrial membrane permeabilization, or apoptosis can also be employed in the invention, as well as peptides that are at least 10-20, 20-

30, 30-50, 50-100, and 100-365 amino acids in size. DNA fragments encoding these polypeptides and peptides are encompassed by the invention. Flanking residues should not disrupt the helical structures described above.

The Vpr variants and other viral apoptotic peptides can be assessed for their ability to mediate apoptosis, and thus their suitability for use as pTox in the invention. It is understood that many techniques could be used to assess binding of Vpr or another viral apoptotic peptide to ANT, and that these embodiments in no way limit the scope of the invention. For example, in one embodiment, surface plasmon resonance is used to assess binding of Vpr or another viral apoptotic peptide to ANT. In another embodiment, electrophysiology is used to assess binding of Vpr or another viral apoptotic peptide to ANT. In another embodiment, purified mitochondria are used to assess binding of Vpr or another viral apoptotic peptide to ANT. In another embodiment, synthetic proteoliposomes are used to assess binding of Vpr or another viral apoptotic peptide to ANT. In another embodiment, microinjection of live cells is used to assess binding of Vpr or another viral apoptotic peptide to ANT. These techniques are described in U.S. Provisional Application No. 60/231,539.

In another embodiment, the yeast two-hybrid system developed at SUNY (described in U.S. Patent No. 5,282,173 to Fields et al.; J. Luban and S. Goff., *Curr Opin. Biotechnol.* 6:59-64, 1995; R. Brachmann and J. Boeke, *Curr Opin. Biotechnol.* 8:561-568, 1997; R. Brent and R. Finley, *Ann. Rev. Genet.* 31:663-704, 1997; P. Bartel and S. Fields, *Methods Enzymol.* 254:241-263, 1995) can be used to screen for Vpr-ANT interaction as follows. Vpr, or portions thereof, or another viral apoptotic peptide, responsible for interaction, can be fused to the Gal4 DNA binding domain and introduced, together with an ANT molecule fused to the GAL 4 transcriptional activation domain, into a strain that depends on GAl4 activity for growth on plates lacking histidine. Interaction of the Vpr polypeptide or another viral apoptotic peptide with an ANT molecule allows growth of the yeast containing both molecules and allows screening for the molecules that inhibit or alter this interaction (i.e., by inhibiting or augmenting growth). In an alternative embodiment, a detectable marker (e.g. β-galactosidase) can be used to measure binding in a yeast two-hybrid assay.

Alternatively, the binding properties of Vpr peptide fragments or another viral apoptotic peptide can be determined by analyzing the binding of Vpr peptide fragments or another viral

apoptotic peptide to ANT-expressing cells by FACS analysis. This allows the characterization of the binding of the peptides, and the discrimination of relative abilities of the peptide to bind to ANT. *In vitro* binding assays with Vpr or another viral apoptotic peptide can similarly be used to characterize ANT binding activity.

In another specific embodiment, a cytotoxic conjugate of the invention includes an adenine nucleotide translocation (ANT)-derived pro-apoptotic peptide. The pro-apoptotic portion (pTox) of the conjugate can contain, for example, the sequence DKRTQFWRYFPGN (hANT₂104-116[A114P]) or a pseudo-peptidic variant such as [DKRTQFWRYFPGN].

In another specific embodiment, a cytoprotective conjugate of the invention includes ANT-derived anti-apoptotic peptides. The anti-apoptotic portion (pSave) of the conjugate can contain, for example, the sequence DKRTQFWRYFAGN (hANT₂104-116), the sequence LASGGAAGATSLCFVYPL (ANT 117-134) or a pseudo-peptidic variant such as D[DKRTQFWRYFPGN].

The pTarg component of the chimeric polypeptide of the invention can be an antibody or an antibody fragment. The antibody or antibody fragment can be all or part of a polyclonal or monoclonal antibody. The term "antibodies" is meant to include polyclonal antibodies, monoclonal antibodies, fragments thereof, as well as any recombinantly produced binding partners. Antibodies are defined to be specifically binding if they bind with a K_a or greater than or equal to about 10⁷ M⁻¹. Affinities of binding partners or antibodies can be readily determined using conventional techniques, for example those described by Scatchard *et al.*, *Ann. N.Y. Acad. Sci.*, 51:660 (1949).

As used herein, the term "antibody fragment" includes the following:

| Fc | A constant region dimer lacking C _H 1 |
|---------|--|
| Fab | A light chain dimerized to V _H -C _H 1 resulting from papain cleavage; this is monomeric since papain cuts above the hinge cystines |
| F(ab)'2 | A dimer of Fab' resulting from pepsin cleavage below the hinge disulfides; this is bivalent and can precipitate antigen |

| Fab' | A monomer resulting from mild reduction of F(ab)' ₂ : an Fab with part of the hinge |
|------|--|
| Fd | The heavy chain portion of Fab (V _H -C _H 1) obtained following reductive denaturation of Fab |
| Fv | The variable part of Fab: a V _H -V _L dimer |
| Fb | The constant part of Fab: a C _H 1-C _L dimer |
| pFc' | A C _H 3 dimer |

Fragments of monoclonal antibodies are of particular interest as small antigen targeting molecules. Antibody fragments are also useful for the assembly of the chimeric polypeptides of the invention designed to carry other pTox agents, such as a therapeutic conjugate. For *in vivo* applications, fragments of antibodies are of interest due to their altered pharmacokinetic behavior, which is useful for cancer therapy with cytotoxic agents, and for their rapid penetration into body tissues, which offer advantages for therapy techniques.

An antibody fragment of particular interest for use in the invention is a minimal Fv fragment with antigen-binding activity. The two chains of the Fv fragment are less stably associated than the Fd and light chain of the Fab fragment with no covalent bond and less non-covalent interaction, but nevertheless functional Fv fragments have been expressed for a number of different antibodies. Two strategies can be employed to stabilize the Fv fragments used in the invention: firstly, mutating a selected residue on each of the V_H and V_L chains to a cysteine to allow formation of a disulphide bond between the two domains; and secondly, the introduction of a peptide linker between the C-terminus of one domain and the N-terminus of the other, such that the Fv is produced as a single polypeptide chain known as a single-chain Fv.

Thus, single-chain Fvs (ScFvs), recombinant V_L and V_H fragments covalently tethered together by a polypeptide link and forming one polypeptide chain, are useful in this invention. For expression of Fv genes, several systems can be effectively used, including myeloma cells, insect, yeast, and *Escherichia coli* cells. Expression in *E. coli* has been a frequently used production method, with both intracellular expression and secretion enabling high yields of ScFv to be made.

The production of ScFv molecules requires the identification of a suitable peptide linker to span the 35-40 Å distance between the C-terminus of one domain and the N-terminus of the other and allow correct folding and assembly of the Fv structure. Several different types of linkers have been used and shown to result in functional ScFv. Polypeptides with the average length of 3-18 amino acids are usually used as links. They can be rich in serine and/or glycine residues, which introduce flexibility, or in charged glutamic acid and/or lysine residues, which improve solubility. Linkers can be selected from searching existing protein structures for protein fragments of the appropriate length and conformation, or by designing them *de novo* based on simple, flexible structures, such as the 15 amino acid sequence (Gly₄Ser)₃.

Active single-chain Fv molecules in both of the two possible orientations, V_H -linker- V_L or V_L -linker- V_H are useful in the invention; however, for some antibodies one particular orientation may be preferable as a free N-terminus of one domain, or C-terminus of the other, may be required to retain the native conformation and thus full antigen binding.

The ScFv may be susceptible to aggregation, with dimers, trimers, and multimers formed. The potential of forming dimers or other multimers with very short linkers, or no linker at all, can be exploited to produce stable pTarg structures. Such an approach can also be used to create pTarg molecules with two different binding specificities by fusing the V_H of an antibody of one specificity to the V_L of another and vice versa.

Fv's stabilized by disulphide linkages can also be employed as the pTarg component of the chimeric polypeptide of the invention. The introduction of a disulphide bond between the V_H and V_L domains to form a disulphide-linked Fv requires the identification of residues in close proximity on each chain, which are unlikely to affect directly the conformation of the binding site when mutated to cysteine, and will be capable of forming a disulphide bond without introducing strain into the structure of the Fv. Sites have been identified in both CDR regions and framework regions, which appear to result in the formation of such disulphide bonds and allow the production of stabilized Fv fragments which retain antigen-binding characteristics.

Due to small size, rapid clearance *in vivo*, stability, and easy engineering, ScFvs employed in this invention have various applications in the treatment of diseases, particularly of cancer. ScFvs can exhibit the same affinity and specificity for antigen as monoclonal antibodies. Dozens of ScFvs with different specificities have been constructed. They are useful for genetic

fusion to the potent toxins (pTox). If the monovalency of ScFv is a disadvantage, constructs with di- or multivalency with increased combining efficiency can be employed.

In a preferred embodiment of the invention, the targeting part (pTarg) of the cytotoxic conjugate is a recombinant portion (ScFv) of a tumor specific antibody, such as the ScFv versions of the M350 and V461 monoclonal antibodies. The hybridoma has been deposited at the CNCM on January 24, 2001, under the Accession Number I-2617.

The pTarg component of the chimeric polypeptide of the invention is preferably a monoclonal antibody or a fragment thereof. Monoclonal antibodies to human cell antigens are preferred. Many tumor-associated antigens are now known and characterized, and antibodies to these allow targeting to different tumor types. Useful tumor-associated antigens are absent on normal tissues and present at high levels on tumor cells, preferably homogeneously on all cells of the tumor. Antigen should also not be shed from the tumor into the blood.

Commonly used tumor-associated antigens and examples of antibodies raised against them are described in the following Table.

| Antigen | Tumor type | Representative antibody |
|--|--|-------------------------|
| Turmor-associated glycoprotein 72 (TAG72), 72 kDa glycoprotein | Pancarcinoma | B72.3, CC49 |
| Carcinoembryonic antigen (CEA), 180 kDa blycoprotein | Pancarcinoma | NP-4, A5B7 |
| Polymorphic epithelial mucin (PEM), >100 kDa glycoprotein | Ovarian, breast, lung | HMFG1 |
| Epithelial membrane antigen (EMA), 40 kDa glycoprotein | Colorectal (and other epithelial tumors) | 17-1A |
| epidermal growth factor receptor (EGFR), 175 kDa glycoprotein | Breast, lung | 425 |
| p185 ^{HER2} /c-erb-B2 | | |

| Antigen | Tumor type | Representative antibody |
|---|----------------------------|-------------------------|
| (185 kDa glycoprotein) | Breast, lung | 4D5 |
| Prostate-specific membrane antigen (PSMA), 100 kDa glycoprotein | Prostrate | 7E11-C5.3 |
| CD33 67 kDa glycoprotein | Myeloid leukemia | P67.6,M195 |
| CD 20 35 kDa glycoprotein | Lymphoma | C2B8 |
| GD2 ganglioside | Melanoma, neuroblastoma | 14-18 |

An important consideration is the absolute amount of antibody localized to the tumor site. Therefore, the ideal molecule would localize to the tumor in large amounts, delivering a high dose of pTox while clearing rapidly from the circulation and the rest of the body, minimizing non-specific toxicity. Intact antibodies typically circulate for a long period of time and accumulate high levels of activity at the tumor site, whereas antibody fragments clear more rapidly, sparing the dose to normal tissues.

The antibody fragments can also be prepared by phage-display technology. Phage display is a selection technique, according to which an antibody fragment (ScFv) is expressed on the surface of the filamentous phage fd. For this, the coding sequence of the antibody variable genes is fused with the gene that encoded the minor coat phage protein III (g3p) located at the end of the phage particle. The fused antibody fragments are displayed on the virion surface and particles with the fragments can be selected by adsorption on insolubilized antigen (panning). The selected particles are used after elution to reinfect bacterial cells. The repeated rounds of adsorbtion and infection lead to enrichment. Bacterial proteases can cleave the bond between the g3p protein and antibody fragments, which results in the production of soluble antibody fragments by infected bacterial cells. To release the soluble ScFvs, an excision of the g3p gene is made or an amber stop codon between the antibody gene and the g3p gene is engineered.

Immunoglobins and certain variants thereof are known and many have been prepared in recombinant cell culture. For example, see U.S. Patent 4,745,055; EP 256,654; Faulkner *et al.*, Nature 298:286 (1982); EP 120,694; EP 125,023; Morrison, J. Immun. 123:793 (1979); Köhler *et al.*, P.N.A.S. USA 77:2197 (1980); Raso *et al.*, Cancer Res. 41:2073 (1981); Morrison *et al.*, Ann. Rev. Immunol. 2:239 (1984); Morrison, Science 229:1202 (1985); Morrison *et al.*, P.N.A.S. USA 81:6851 (1984); EP 255,694; EP 266,663; and WO 88/03559. Reassorted immunoglobulin chains also are known. See for example U.S. patent 4,444,878; WO 88/03565; and EP 68,763 and references cited therein. DNA encoding immunoglobulin light or heavy chain constant regions is known or readily available from cDNA libraries or is synthesized. See for example, Adams *et al.*, Biochemistry 19:2711-2719 (1980); Gough *et al.*, Biochemistry 19:2702-2710 (1980); Dolby *et al.*, P.N.A.S. USA, 77:6027-6031 (1980); Rice *et al.*, P.N.A.S. USA 79:7862-7865 (1982); Falkner *et al.*, Nature 298:286-288 (1982); and Morrison *et al.*, Ann. Rev. Immunol. 2:239-256 (1984). These materials and techniques can be employed to synthesize the pTarg component of the chimeric polypeptide of the invention.

Polyclonal antibodies employed as the pTarg component of the chimeric polypeptide of the invention can be readily generated from a variety of sources, for example, horses, cows, goats, sheep, dogs, chickens, rabbits, mice, or rats, using procedures that are well known in the art. In general, purified cell surface proteins or glycoproteins or a peptide based on the amino acid sequence of cell surface proteins or glycoproteins that is appropriately conjugated is administered to the host animal typically through parenteral injection. The immunogenicity of cell surface proteins or glycoproteins can be enhanced through the use of an adjuvant, for example, Freund's complete or incomplete adjuvant. Following booster immunizations, small samples of serum are collected and tested for reactivity to cell surface proteins or glycoproteins. Examples of various assays useful for such determination include those described in *Antibodies: A Laboratory Manual*, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988; as well as procedures, such as countercurrent immuno-electrophoresis (CIEP), radioimmunoassay, radio-immunoprecipitation, enzyme-linked immunosorbent assays (ELISA), dot blot assays, and sandwich assays. See U.S. Patent Nos. 4,376,110 and 4,486,530.

Monoclonal antibodies employed as the pTarg component can be readily prepared using well known procedures. See, for example, the procedures described in U.S. Patent Nos. RE

32,011, 4,902,614, 4,543,439, and 4,411,993; Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses, Plenum Press, Kennett, McKearn, and Bechtol (eds.), 1980. Briefly, the host animals, such as mice, are injected intraperitoneally at least once and preferably at least twice at about 3 week intervals with isolated and purified cell surface proteins or . glycoproteins, conjugated cell surface proteins or glycoproteins, optionally in the presence of adjuvant. Mouse sera are then assayed by conventional dot blot technique or antibody capture (ABC) to determine which animal is best to fuse. Approximately two to three weeks later, the mice are given an intravenous boost of cell surface proteins or glycoproteins or conjugated cell surface proteins or glycoproteins. Mice are later sacrificed and spleen cells fused with commercially available myeloma cells, such as Ag8.653 (ATCC), following established protocols. Briefly, the myeloma cells are washed several times in media and fused to mouse spleen cells at a ratio of about three spleen cells to one myeloma cell. The fusing agent can be any suitable agent used in the art, for example, polyethylene glycol (PEG). Fusion is plated out in plates containing media that allows for the selective growth of the fused cells. The fused cells can then be allowed to grow for approximately eight days. Supernatants from resultant hybridomas are collected and added to a plate that is first coated with goat anti-mouse Ig. Following washes, a label, such as 125I-labeled cell surface proteins or glycoproteins, is added to each well followed by incubation. Positive wells can be subsequently detected by autoradiography. Positive clones can be grown in bulk culture and supernatants are subsequently purified over a Protein A column (Pharmacia).

The monoclonal antibodies for the pTarg component can be produced using alternative techniques, such as those described by Alting-Mees *et al.*, "Monoclonal Antibody Expression Libraries: A Rapid Alternative to Hybridomas", *Strategies in Molecular Biology* 3:1-9 (1990), which is incorporated herein by reference. Similarly, binding partners can be constructed using recombinant DNA techniques to incorporate the variable regions of a gene that encodes a specific binding antibody. Such a technique is described in Larrick *et al.*, *Biotechnology*, 7:394 (1989).

The monoclonal antibodies and fragments thereof employed as the pTarg component include chimeric antibodies, e.g., humanized versions of murine monoclonal antibodies. Such humanized antibodies may be prepared by known techniques, and offer the advantage of reduced immunogenicity when the antibodies are administered to humans. In one embodiment, the

humanized monoclonal antibody comprises the variable region of a murine antibody (or just the antigen binding site thereof) and a constant region derived from a human antibody. Alternatively, a humanized antibody fragment may comprise the antigen binding site of a murine monoclonal antibody and a variable region fragment (lacking the antigen-binding site) derived from a human antibody. Procedures for the production of chimeric and further engineered monoclonal antibodies include those described in Riechmann *et al.* (*Nature* 332:323, 1988), Liu *et al.* (*PNAS* 84:3439, 1987), Larrick *et al.* (*Bio/Technology* 7:934, 1989), and Winter and Harris (*TIPS* 14:139, May 1993). Procedures to generate antibodies transgenically can be found in GB 2,272,440, US Patent Nos. 5,569,825 and 5,545,806 and related patents claiming priority therefrom, all of which are incorporated by reference herein.

In a further embodiment of the invention, the targeting part (pTarg) of a cytotoxic chimeric polypeptide is a tumor homing peptide. Such a tumor homing peptide include any homing sequence described by Ellerby *et al.*, in example V, VI, VII, VIII of PCT/US00/01602, the entire disclosure of which is relied upon and incorporated by reference herein.

In preferred embodiments of the invention, the chimeric polypeptide has the sequence CNGRCGG-HFRIGCRHSRIG, or CNGRCGG-D[HFRIGCRHSRIG], or CNGRCGG-Vpr52-96, or CNGRCGG-DKRTQFWYFPGN, or CNGRCGG-D[DKRTQFWYFPGN], or ACDCRGDCFCGG-HFRIGCRHSRIG, or ACDCRGDCFCGG-D[HFRIGCRHSRIG], or ACDCRGDCFCGG-Vpr52-96, or ACDCRGDCFCGG-DKRTQFWYFPGN, or ACDCRGDCFCGG-[DKRTQFWYFPGN], or M350/ScFv-HFRIGCRHSRIG, or M350/ScFv-D[HFRIGCRHSRIG] or M350/ScFv-Vpr52-96, or M350/ScFv-DKRTQFWYFPGN, or or M350/ScFv-D[DKRTQFWYFPGN].

Chimeric polypeptides of the invention can be generated by a variety of conventional techniques. Such techniques include those described in B. Merrifield, Methods Enzymol, 289:3-13, 1997; H. Ball and P. Mascagni, Int. J. Pept. Protein Res. 48:31-47, 1996; F. Molina *et al.*, Pept. Res. 9:151-155, 1996; J. Fox, Mol. Biotechnol. 3:249-258, 1995; and P. Lepage *et al.*, Anal. Biochem. 213: 40-48, 1993.

Peptides can be synthesized on a multi-channel peptide synthesizer using classical Fmocbased and pseudopeptide synthesis. In one embodiment of the invention, Vpr52-96, Vpr71-96 and Vpr 71-82 and all the Tox, Save and TARG peptides described in Table I, II, III, are

synthesized by solid phase peptide chemistry. After cleavage from the resin, the peptides are purified and analyzed by reverse-phase HPLC. The purity of the peptides is typically above 98% according to HPLC trace. The integrity of each peptide can be controlled by matrix Assisted Laser Desorption Time of Flight spectrometry. To avoid rapid degradation of the peptides in biological fluids, one or several amide bonds could be advantageously replaced by peptide bond isosters like retro-inverso (NH-CO), methylene amino (CH₂-NH), carba (CH₂-CH₂) or carbaza (CH₂-CH₂-N(R)) bonds.

Alternatively, the chimeric polypeptides of the invention can be prepared by subcloning a DNA sequence encoding a desired peptide sequence into an expression vector for the production of the desired peptide. The DNA sequence encoding the peptide is advantageously fused to a sequence encoding a suitable leader or signal peptide. Alternatively, the DNA fragment may be chemically synthesized using conventional techniques. The DNA fragment can also be produced by restriction endonuclease digestion of a clone of, for example HIV-1, DNA using known restriction enzymes (New England Biolabs 1997 Catalog, Stratagene 1997 Catalog, Promega 1997 Catalog) and isolated by conventional means, such as by agarose gel electrophoresis.

In another embodiment, the well known polymerase chain reaction (PCR) procedure can be employed to isolate and amplify a DNA sequence encoding the desired protein or peptide fragment. Oligonucleotides that define the desired termini of the DNA fragment are employed as 5' and 3' primers. The oligonucleotides can contain recognition sites for restriction endonucleases, to facilitate insertion of the amplified DNA fragment into an expression vector. PCR techniques are described in Saiki *et al.*, Science 239:487 (1988); Recombinant DNA Methology, Wu *et al.*, eds., Academic Press, Inc., San Diego (1989), p. 189-196; and PCR Protocols: A Guide to Methods and Applications, Innis *et al.*, eds, Academic Press., (1990). It is understood of course that many techniques could be used to prepare polypeptide and DNA fragments, and that this embodiment in no way limits the scope of the invention.

Several methods can be used to link TARG to TOX and TARG to SAVE, depending on the particular chemical characteristics of the molecules. For example, methods of linking haptens to carrier proteins as used routinely in the field of applied immunology. In one embodiment, a premade a PTPC regulatory molecule (TOX or SAVE) can be conjugated to an antibody as antibody fragment (pTarg) using, for example, carbodiimide conjugation.

Carbodiimides comprise a group of compounds that have the general formula R-N+C=N-R, where R and R can be aliphatic or aromatic, and are used for synthesis of peptide bonds. The preparative procedure is simple, relatively fast, and is carried out under mild conditions. Cardodiimide compounds attack carboxylic groups to change them into reactive sites for free amino groups. Carbondiimide conjugation has been used to conjugate a variety of compounds for the production of antibodies.

The water soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) can be useful for conjugating a PTPC regulatory molecule (TOX or SAVE) to an antibody or antibody fragment molecule. Such conjugation requires the presence of an amino group, which can be provided, for example, by a PTPC regulatory molecule (TOX or SAVE), and a carboxyl group, which can be provided by an antibody or antibody fragment.

In addition to using carbodiimides for the direct formation of peptide bonds, EDC also can be used to prepare active esters, such as N-hydroxysucinimide (NHS) ester. The NHS ester, which binds only to amino groups, then can be used to induce the formation of an amide bond with the single amino group of the oxorubicin. The use of EDC and NHS in combination is commonly used for conjugation in order to increase yield of conjugate formation.

Other methods for conjugating a PTPC regulatory molecule (TOX or SAVE) to an antibody or antibody fragment also can be used. For example, sodium periodate oxidation followed by reductive alkylation of appropriate reactants can be used, as can glutaraldehyde crosslinking. However, it is recognized that, regardless of which method of producing a chimeric polypeptide of the invention is selected, a determination must be made that an antibody or antibody fragment maintains its targeting ability and that a PTPC regulatory molecule (TOX or SAVE) maintains its activity.

The chimeric polypeptide of the invention may further incorporate a specifically non-cleavable or cleavable linker peptide functionally interposed between the PTPC regulatory molecule (TOX or SAVE) (pTarg) and the antibody or antibody fragment (pTox). Such a linker peptide provides by its inclusion in the chimeric construct, a site within the resulting chimeric polypeptide that may be cleaved in a manner to separate the intact PTPC regulatory molecule (TOX or SAVE) from the intact antibody or antibody fragment. Such a linker peptide may be, for instance, a peptide sensitive to thrombin cleavage, factor X cleavage, or other peptidase

cleavage. Alternatively, where the chimeric polypeptide lacks methionine, the antibody or antibody fragment may be separated by a peptide sensitive to cyanogen bromide treatment. In general, such a linker peptide will describe a site, which is uniquely found within the linker peptide, and is not found at any location in either of the TARG, TOX or SAVE fragment constituting the chimeric polypeptide.

Compositions comprising an effective amount of a chimeric polypeptide of the present invention, in combination with other components, such as a physiologically acceptable diluent, carrier, or excipient, are provided herein. The chimeric polypeptide can be formulated according to known methods used to prepare pharmaceutically useful compositions. They can be combined in admixture, either as the sole active material or with other known materials suitable for a given indication, with pharmaceutically acceptable diluents (e.g., saline, Tris-HCl, acetate, and phosphate buffered solutions), preservatives (e.g., thimerosal, benzyl alcohol, parabens), emulsifiers, solubilizers, adjuvants and/or carriers. Suitable formulations for pharmaceutical compositions include those described in *Remington's Pharmaceutical Sciences*, 16th ed. 1980, Mack Publishing Company, Easton, PA.

In addition, such compositions can be complexed with polyethylene glycol (PEG), metal ions, or incorporated into polymeric compounds such as polyacetic acid, polyglycolic acid, hydrogels, dextran, etc., or incorporated into liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts or spheroblasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance, and are thus chosen according to the intended application.

The compositions of the invention comprising the chimeric polypeptide can be administered in any suitable manner, e.g., topically, parenterally, or by inhalation. The term "parenteral" includes injection, e.g., by subcutaneous, intravenous, or intramuscular routes, also including localized administration, e.g., at a site of disease or injury. Sustained release from implants is also contemplated. One skilled in the pertinent art will recognize that suitable dosages will vary, depending upon such factors as the nature of the disorder to be treated, the patient's body weight, age, and general condition, and the route of administration. Preliminary doses can be determined according to animal tests, and the scaling of dosages for human administration is performed according to art-accepted practices.

Compositions comprising nucleic acids in physiologically acceptable formulations are also contemplated. DNA may be formulated for injection, for example.

In one of its most general applications, the invention relates to a recombinant vector incorporating a DNA segment having a sequence encoding the chimeric polypeptide of the invention. For the purposes of the invention, the term "chimeric polypeptide" is defined as including any polypeptide where at least a portion of a viral apoptotic peptide is coupled to at least a portion of an antibody or antibody fragment. The coupling can be achieved in a manner that provides for a functional transcribing and translating of the DNA segment and message derived therefrom, respectively.

The vectors of the invention will generally be constructed such that the chimeric polypeptide encoding sequence is positioned adjacent to and under the control of an effective promoter. In certain cases, the promotor will comprise a prokaryotic promoter where the vector is being adapted for expression in a prokaryotic host. In other cases, the promoter will comprise a eukaryotic promoter where the vector is being adapted for expression in a eukaryotic host. In the later cases, the vector will typically further include a polyadenylation signal position 3' of the carboxy-terminal amino acid, and within a transcriptional unit of the encoded chimeric polypeptide. Promoters of particular utility in the vectors of the invention are cytomegalovirus promoters and baculovirus promoters, depending upon the cell used for expression. Regardless of the exact nature of the vector's promoters, the recombinant vectors of the invention will incorporate a DNA segment as defined below.

A recombinant host cell is also claimed herein, which incorporates a vector of the invention. The recombinant host cell may be either a eukaryotic cell or a prokaryotic host cell. Where a eukaryotic cell is used, a Chinese Hamster Ovary (CHO) cell has utility. In another embodiment, when used in combination with a baculovirus promoter, the insect cell lines SF9 or SF21 can be used.

This invention will be described in greater detail in the following Examples.

EXAMPLE 1

Obtaining the murine monoclonal antibody (Ac M350)

Human fetal cells were chosen as a source of immunization. It was the well-known similarities between fetal and tumoral antigens which inspired us to use fetal cells as a source of immunization to produce monoclonal antibodies directed against the epitopes present on tumoral cells. Oncofetal antigens are glycoproteins which are present during intra-uterine life; they disappear at birth and can be re-expressed in pathological situations, particularly in malignant tumors. There are many examples of this antigen community, the best known models being fetoprotein which is associated with 70% of liver tumors, and <<embryo tumor antigens>>, which is often used in human clinical practice and which is a monitoring parameter for patients suffering from cancers of the digestive tract.

A. M350 clone production

These fetal cells were obtained from the sterile removal of the mammary buds of 25-week old female fetuses. Once the buds had been mechanically dissociated into 0.5 mm³ fragments, the cells were resuspended in a Dulbecco medium modified with collagenase and hyalurodinase at 37°C and shaken for between 30 minutes and 4 hours after being monitored under the microscope. As soon as organoids appear, the cells were deposited onto Ficoll, washed, then cultured in a calcium-free DMEM-F12 medium, in hepes, insulin, choleric toxin, cortisol. Once the cells were subcultured once a week. Using this technique the cells duplicated 10 to 20 times giving sufficient cells for immunization purposes.

Balb/c mice were immunized four times, intraperiotonaly. The fusion was achieved according the classical technic of Kohler and Milstein. The screening was done with fetal mammary cells, adult mammary cells and breast tumors. Several clones appeared and one, M350 clone, was particularly tested on breast tumors and normal breast tissues. 150 tumor sections were tested: (i.e.) infiltrating intra-canalar and intra-lobular adenocarcimonas, infiltrating lobular adenocarcimonas. Tests were performed using an immunoenzymatic technic with alkaline phosphatase. All the tumors tested positive whereas the normal tissues taken from mammary

samples tested in parallel were negative for weakly positive. Each slide of normal tissue contained lobular type epithelial structures and cavities inside the paleal tissue.

B. Other Hybridomes

Obtaining new murine monoclonal antibodies against associated breast tumor antigens.

In this technology, C57/B16 mice were immunized four times, intraperitonally, with a mixture of three different breast tumor cell lines (MCF7, MDA, ZR75-1). After fusion and screening the specificity was studied on normal breast tissues and malignant tumors, other tumor samples and peripheral blood cells. The Monoclonal antibodies showing surface tumor labeling were chosen.

EXAMPLE 2

A Cell lines and viruses

The insert cells derived from ovarian tissue of Spodoptera frugiperda (Sf9 insect cells, Vaughn et coll., 1977) and insect cells derived from Trichoplusia ni (High Five insect cells) were maintained at 28°C in TC100 medium supplemented with 5% fetal calf serum and were used for the propagation of recombinant baculoviruses and for the production of recombinant proteins. The recombinant baculoviruses are obtained after co-transfection of insect cells with baculovirus viral DNA (Baculogold, Pharmingen) and recombinant transfer vector DNA.

B. Recombinant transfer vector: pVL-PS-gp671

The recombinant transfer vector pVL-PSgp671 derived from transfer vector pVL1392 (Invitrogen) is used as transfer vector to generate recombinant viruses. It includes from 5' to 3': the peptide signal sequence of gp67 baculovirus glycoprotein, the sequence coding for a His(6)-Tag, the recognition sequence for the Xa Factor, a polylinker region for subcloning the scFv sequence, a link-sequence: GGC required for the covalent association between cytotoxic peptides and ScFv.

The signal peptide sequence from gp67 was added by insertion of a PCR product of gp67 (obtained by PCR from a commercial pcGP67-B plasmid as a template and the PSgp67-Back and PSgp67-For as primers) at the *Bg*/II site of the pVL1392 plasmid. The sequence coding for the His(6)-Tag sequence and the recognition sequence for the Xa factor were then added by using

insertion of oligonucleotides at the 3' end of the gp67 sequence. By the same way the sequence of the peptide motif required for the covalent association between cytotoxic peptides and ScFv: (-Gly-Gly-Cys) was added at the 3' part of the polylinker (the first G is encoded by the last nucleotide of the Xmal site).

Insertion at BamH1 and Bg1I of overlaping primers:

Th1: GAT CCC ATC ATC ACC ACC ACC AC (BamHI-His(6))

Th2: ATT GAA GGA AGA GAATTC CCATG (Factor Xa cleveage -EcoRI-NcoI)

Th3: GCT GCA GCC CGG GGG ATG TTA AA (Pst1 -XmaI -GGS - STOP- BamHI)

Th4: CTT CCT TCA ATG TGG TGG TGG TGA TGA TGG (link beween Th1 Th2)

Th5: GGG CTG CAG CCA TGG GAA TTC T (link between Th2 and Th3)

Th6: GAT CTT TAA CAT CCC CC (link between Th3 and pVL, -pg67)

C Synthesis of ScFv DNA fragment

VH and VL regions of M350:

Total RNA isolated from M350 hybridome have been used as a template for a reverse transcription using oligo (dT) as primers (Reverse Transcription IBI Fermentas). A PCR realized with those cDNAs and specific primers (mouse Ig-Prime-Kit, Novagen) have led to the selective amplification of VH and VL chains. These regions are then cloned in "blunt" in pST-Blue 1 plasmid and sequenced.

VH and VI. regions of other hybridoines:

Total RNA isolated from selected hybridome was used as a template for a reverse transcription using oligo (dT) (Reverse Transcription IBI Fermentas). A PCR with specific primers (mouse Ig-Prime-Kit, Novagen) led to the selective amplification of VH and VL chains. These products are then cloned in pGEMT (TA cloning System front PROMEGA) vector and sequenced. Three new VH and VL sequences were determined from clone therap.99B3 (Figure 3), clone therap.88E10 (Figure 4), and therap.152C3 (Figure 5).

Obtention of the ScFv-transfer vector:

VH-link-VL chimeric DNA were done by fusion-PCR in two steps (Figure 12). The first

step added a link-sequence (Gly-Gly-Gly-Gly-Ser) at the 3' of the VH chain and at the 5' end of the VL chain respectively. The second step was a PCR fusion leading to the chimeric DNA: VH-link-VL. The set of primers used in this second step brings a 5'-EcoRI and a 3'-XmaI sites to VH and VL respectively that will be used for the subcloning of the final product in pVL-PSgp671 vector (**Figure 13**).

D Cotransfection and purification of recombinant baculoviruses

Sf9 cells were cotransfected with viral DNA (BaculoGold; Pharmingen) and recombinant transfer vector DNA (pVL-PSgp671-ScFv) by the lipofection method (Feloner and Ringold, 1989) (DOTAP; Roche). Screening and purification of recombinant viruses were carried out by the common procedure described by Summers and Smith (Summers and Smith, 1987). The recombinant virus was named BAC-PSgp671-scFv and amplified to constitute a viral stock with an M0I of 10⁸.

E Analysis of recombinant proteins

Infected cells were collected, washed with cold phosphate-buffered saline (PBS) and resuspended in sample reducing buffer (Laemmli, 1970). After boiling (100°C for 5 min), proteins samples were resolved by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions (Laemmli, 1970). The apparent molecular weight of the protein was check by coomassie blue staining or the proteins were transferred onto a nitrocellulose filter (Schleicher and Schuell; BAS 85, 0.45µm) with a semidry blotter apparatus (Ancos). The nitrocellulose membrane was then stained with Ponceau Red (Sigma) and subsequently blocked with a solution of Tris-saline buffer (0.05 M Tris-HCI ph7.4, 0.2 M NaC1) containing 0.05% Tween 20 and 5% non fat milk (TS-sat). ScFv was detected using a mouse monoclonal antibody raised against His(6)-Tag (SIGMA) as primary antibody and a sheep anti-mouse immunoglobulin G (IgG)- horseradish peroxydase conjugate as secondary antibody (1; 3000 Amersham). The immunoreactive bands were visualized by using ECL reagents as described by the manufacturer (Amersham).

F Protein production and purification

To obtain viral stock, Sf9 insect cells cultured in IPL41 medium and 5% FCS are infected in exponential phase with the recombinant baculoviruses at MOI1. After a 7-day incubation period at 28° in IPL41 medium with 5% FCS, the supernatant is harvested by centrifugation at 8000 RPM during 15 min. Then High-five insect cells cultured in Xpress media (Biowhitaker) are infected with recombinant baculovirus in exponential phase at MOI 10, following lh30 of infection High Five cells were harvested by centrifugation and resuspended in Xpress media without serum. After a 4-day period of incubation at 28°C, the supenatant is harvested by centrifugation at 8000 RPM during 15 min. These supernatants are then concentrated by two rounds of ammonium sulfate precipitation. The precipitate obtained by sedimentation is dialyzed during 12 hours and purified using batch of Ni-NTA agarose beads as described by the manufacturer (Qiagen). After dialysis (2 days, PBS, 4°C) and analysis by Coomassic staining purified proteins were used for the covalent association with cytotoxic peptides.

EXAMPLE 3

Method of coupling ScFv to pTox

The peptide was assembled using Fmoc solid phase peptide synthesis, after the last Fmoc deprotection a propionyloxy succinimide ester was allowed to react, in the presence of diisopropyl ethylamine, with the alpha amino group of the peptide. At the end of the reaction (30 min) the peptide resin was washed with methylene chloride and the peptide was classically cleaved and deprotected under acidic conditions. The activated peptide was then purified by HPLC and its integrity was confirmed by mass spectrometry. The activated peptide was then allowed to react with the ScFv with peptide in a molar ratio of 10:1 (pH7, PBS, glass tube over agitation for 3 hours at room temperature). Then, dialysis was done for 48h against PBS a 4°C. Four Tox peptides were coupled to ScFv using this method:

Tox 11 ScFv-M350-Jac5 (Vpr71-96[C761])

Ctr1ToX11I ScFv-M350-Jac5M (Vpr71-96[C76S;R73,80A])

Tox 12 ScFv-Vpr52-96[C76S]

Ctr1Tox12 ScFv - Vpr52-96[C76S; R73A; R80A]

EXAMPLE 4

Examples of Targ-Tox or Targ-Save structures

All the Tox peptides can have a facultative N-terminal biotin and a facultative C-terminal amide fonction. Tox0 is a Tox peptide which does not necessarily require an association with a Targ. Tox1, Tox2, Tox 5, Tox6, Save1, Save2 and their respective control can posses a facultative gly-gly- (-GG-) linker between the Targ and the Tox/Save motif.

| Tox0 | Biot-DTWTGVEALIRILQQLLFIHFRIGCRHSRIGIIQQRRTRNGASKS |
|----------|--|
| Ctr1Tox0 | Biot-DTWTGVEALIRILQQLLFHFAIGCRHSAIGIIQQRRTRNGASKS |

| Tox1 | Biot- CNGRC-GG-HFRIGCRHSRIG |
|----------|-----------------------------------|
| Ctr1Tox1 | Biot- CNGRC-GG-HFAIGCRHSAIG |
| Ctr2Tox1 | Biot-CNGRC-GG-CNGRC |
| Ctr3Tox1 | Biot-GG-HFRIGCRHSRIG |
| Ctr4Tox1 | Biot-CNGRC-GG-Scramble |
| Ctr5Tox1 | Biot-KETWWETWWTEW-GG-HFRIGCRHSRIG |

| Tox2 | Biot-ACDCRGDCFC-GG-HFRIGCRHSRIG |
|----------|----------------------------------|
| Ctr1Tox2 | Biot- ACDCRGDCFC-GG-HFAIGCRHSAIG |

Tox5

| Tox5 | Biot-CNGRC-GG-DKRTQFWRYFPGN (hANT2m) | |
|----------|--------------------------------------|---|
| Ctr1Tox5 | Biot-CNGRC-GG-DKRTQFWRYFAGN (hANT2) | |
| Ctr2Tox5 | Biot-CNGRC-GG-DRHKQFWRYFPGN (hANT1m) | |
| Ctr3Tox5 | Biot-CNGRC-GG-DKHTQFWRYFPGN (hANT3m) | *************************************** |
| Ctr4Tox5 | Biot-GG-DKRTQFWRYFPGN (hANT2m) | |
| Ctr5Tox5 | Biot-GG-DRHKQFWRYFPGN (hANT1m) | |
| Ctr6Tox5 | Biot-GG-DKHTQFWRYFPGN (hANT3m) | |
| Ctr7Tox5 | Biot-CNGRC-GG-Scramble | |

Tox6

| Tox6 | Biot-ACDCRGDCFC-GG-DKRTQFWRYFPGN (hANT2m) |
|----------|---|
| Ctr1Tox6 | Biot-ACDCRGDCFC-GG-DKRTQFWRYFAGN (hANT2) |
| Ctr2Tox6 | Biot-ACDCRGDCFC-GG-DRHKQFWRYFPGN (hANT1m) |
| Ctr3Tox6 | Biot-ACDCRGDCFC-GG-DKHTQFWRYFPGN (hANT3m) |
| Ctr4Tox6 | Biot-ACDCRGDCFC-GG |
| Ctr5Tox6 | Biot-ACDCRGDCFC-GG-Scramble |

| Tox 11 | ScFv-M350-Jac5(Vpr71-96[C76]) |
|-----------|--|
| Ctr1Tox11 | ScFv-M350-Jac5M(Vpr71-96[C76;R73,80A]) |

Save1

| Save1 | Biot-RKKRRQRRR-DKRTQFWRYFAGN (hANT2) |
|-----------|---------------------------------------|
| Ctr1Save1 | Biot-RKKRRQRRR-DKRTQFWRYFPGN (hANT2m) |
| Ctr2Save1 | Biot-RKKRRQRRR-DRHKQFWRYFAGN (hANT1) |
| Ctr3Save1 | Biot-RKKRRQRRR-DKHTQFWRYFAGN (hANT3) |
| Ctr4Save1 | Biot-RKKRRQRRR |
| Ctr5Save1 | Biot-RKKRRQRRR-Scramble |

Save2

| Save 2 | Biot-RKKRRQRRR-LASGGAAGATSLCFVYPL (hANT[117-134]) |
|-----------|--|
| Ctr1Save2 | Biot-RKKRRQRRR-GAWSNVLRGMGGAFVLVLY (ANTTM6[271-289]) |
| Ctr2Save2 | Biot-RKKRRQRRR-scramble |

EXAMPLE 5

Evaluation of mitochondrial and nuclear parameters of Apoptosis in cells (cell lines) and cell-free systems

A. Cells

MCF-7, MDA-MB231, COS and HeLa cells are cultured in complete culture medium (DMEM supplemented with 2 mM glutamine, 10% FCS, 1 mM Pyruvate, 10 mM Hepes and 100 U/ml pencillin/streptomycin). Jurkat cells expressing CD4 and stably transfected with the human Bcl-2 gene or a Neomycin (Neo) resistance vector [Aillet, *et al.*, 1998 J. Virol. 72:9698-9705] only were kindly provided by N. Israel (Pasteur Institute, Paris). Neo and Bcl-2 U937 cells [Zamzami *et al.*, 1995 J. Exp. Med], and CEM-C7 cells are cultured in RPMI 1640 Glutamax medium supplemented with 10% FCS, antibiotics, and 0.8 μg/ml G418.

The cell **tests** that have been implemented determine the pathway (intracellular penetration, then subcellular localization) of the candidates, and the apoptotic status ($\Delta \psi m$, activation and relocalization of cell death effectors, content in nuclear DNA) of the target cell. In order to determine these parameters it is necessary to use fluorescent probes to label the cells and/or the candidates molecules and to implement the following two analytical procedures: multi-parameter cytofluorimetry and fluorescent microscopy. As far as neuroprotection is

concerned, tests were carried out on primary cultures of cortical neuronal cells from mice embryos. As far as cardioprotection is concerned, tests were carried out on primary cultures of cardiomyocytes from mice embryos.

- Intra-cellular pathway tests: the TARG-TOX ou TARG-SAVE peptides coupled either with biotin (detected using fluorochromes conjugated with streptavidin; or by ligand-blot after subcellular fractioning) or with FITC (detected by direct observation of living cells, videomicroscopy and image analysis) are added to the cells. It possible to favor the TOX or SAVE mitochondrial routing by inserting mitochondrial addressing signals (the Apoptosis Inducing Factor or ornithin transcarbamylase, for example). Similarly, the mitochondrial routing is evaluated after modifying sequences and certain lateral chains (phosphorylations, methylations), then replacing the peptides by peptidomimetics.
- Multi-parameter analysis of apoptosis on tumoral and endothelial cell lines, and primary neurons. Fluorescents probes wil be used to mesure the state of the mitochondrial transmembrane potential (JCI, DioC6, mitoTrackers) and nuclear condensation (Hoescht). Similarly, the post-mitochondrial parameters of apoptosis are evaluated using classical hypoploidy tests and cell surface labeling with annexin V-FITC.

In this type of tests, we evaluate either the cytotoxic potential of the TARG-TOX, i.e. their capacity to kill (via a mitochondrial effect) tumoral ou endothelial cell lines (the best TARG-TOX must also kill over-expressing Bel-2 cell lines); or the cytoprotective potential of the TARG-SAVE when the neurons are subjected to different apoptogenic treatments.

B. Apoptosis Modulation

PBS-washed cells (1-5 x 10⁵ /ml) are incubated with (1 to 5 μM) of pTarg-pTox in complete culture medium supplemented or not with cyclosporin A (CsA; 1 μM), bongkrekic acid (BA; 50 μM), and/or the caspase inhibitors N-benzyloxycarbonyl-Val-Ala-Asp.fluoromethylketone (Z-VAD.fmk; 50 μM; Bachem Bioscience, Inc.), Boc-Asp-fluoromethylketone (Boc-D.fmk), or *N*-benzyloxycarbonyl-Phe-Ala-fluoromethylketone (Z-FA.fmk; all used at 100 μM added each 24 h; Enzyme Systems). During exposure to pTarg-pTox, human primary PBLs from healthy donors, purified with Lymphoprep (Pharmacia), are cultured in RPMI 1640 Glutamax medium without any addition of serum. In contrast, PHA blasts (24 h of 1 μg/ml PHA-P [Wellcome Industries]; 48 h with 100 U/ml human recombinant IL-2 [Boehringer Mannheim]) are cultured with 10% FCS.

C. Cytofluorimetric Determinations of Apoptosis-associated Alterations in Intact Cells

For cytofluorometry, the following fluorochromes are employed: 3,3'-dihexyloxacarbocyanine iodide (DiOC(6)3; 40 nM) for mitochondrial transmembrane potential ($\Delta\Psi$ m) quantification, hydroethidine (4 μ M) for the determination of superoxide anion generation, and propidium. iodide (PI; 5 μ M) for the determination of viability (Zamzami, N., *et al.*, 1995. J. Exp. Med. 182:367-377). The frequency of subdiploid cells is determined by PI (50 μ g/ml) staining of ethanol-permeabilized cells treated with 500 μ g/ml RNase (Sigma Chemical Co.; 30 min, room temperature [RT]) in PBS, pH 7.4, supplemented with 5 mM glucose (Nicoletti, I. *et al.*, 1991. J. Immunol. Methods. 139:271-280).

D. Fluorescence staining of life cells and immunofluorescence

For the assessment of mitochondrial and nuclear features of apoptosis, cells cultured on a cover slip are incubated with the $\Delta\Psi$ m-sensitive dyes chloromethyl-X-rosamine (CMXRos; 50 nM; Molecular Probes, Inc.) or 5,5',6,6'-tetrachloro-l,l', 3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, 2 μ M, Molecular Probes), the $\Delta\Psi$ m-insensitive dye Mitotracker green (1 μ M; Molecular Probes, Inc.), and/or Hoechst 33342 (2 μ M, Sigma) for 30 min at 37°C in complete culture medium (.Marzo, let al. 1998. Science. 281:2027-2031).

E. For in situ determinations of pTarg-pTox internalisation

For *in situ* determinations of TARG-(MLS)-TOX/SAVE internalisation, cells are incubated at different times with TARG-(MLS)-TOX/SAVE, and then cells are fixed with 4% paraformaldehyde and 0.19% picric acid in PBS (pH 7.4) for 1 h at RT. Fixed cells are permeabilized with 0.1% SDS in PBS at RT (for 5 min), blocked with 10% FCS, and stained with an mAb specific for hexa-histidine tag (clone HIS-1, IgG2a, SIGMA) revealed by a goat anti-mouse PE conjugate [Southern Biotechnology Associates, Inc.]), Hsp60 (mAb H4149 [Sigma Chemical Co.], revealed by a goat anti-mouse IgG1 FITC conjugate), cytochrome c oxidase (COX; mAb 20E8-C12 [Molecular Probes, Inc.], revealed by a goat anti-mouse IgG2a FITC conjugate), or when the Targ is a biotinylated peptide, a streptavidin-PE reagent is added 30 min. followed by detection of the fluorescence intensity by fluorescence (and/or confocal) microscopy.

F. Assessment of mitochondrial parameters in vitro

Mitochondria are purified from rat liver, as described (Costantini et al., 1996), and resuspended in 250 mM sucrose + 0.1 mM EGTA + 10 mM -tris[hydroxymethyl]methyl-2-Aminoethanesulfonic acid, pH=7.4). For the induction of PT, mitochondria (0.5 mg protein per ml) are resuspended in PT buffer (200 mM sucrose, 10 mM Tris-MOPS (pH 7.4), 5 mM Trissuccinate, 1 mM Tris-phosphate, 2 µM rotenone, and 10 µM EGTA-Tris), and monitored in an F4500 fluorescence spectrometer (Hitachi, Tokyo, Japan) for the 90° light scattering of light (545 nm) to determine large amplitude swelling after addition of 2 mM atractyloside (Atr), 1 µM cyclosporin A (CsA; Novartis, Basel, Switzerland), 5 µM CaCl₂, and/or 0.5 to 20 µM of pTargpTox or pTarg-pSave. For the determination of the $\Delta \Psi_m$, mitochondria (0.5 mg protein per ml) are incubated in a buffer supplemented with 1 µM rhodamine 123 (Molecular Probes, Eugene, OR) and the dequenching of rhodamine fluorescence (excitation 505 nm, emission 525 nm) is measured as described (Shimizu et al., 1998). Supernatants from mitochondria (6800 g for 15min; then 20 000 g for 1 h; 4°C) are frozen at -80°C until determination of apoptogenic activity on isolated nuclei, DEVD-afc cleaving activity, and immunodetection of cytochrome c and AIF. Cytochrome c and AIF are detected by means of a monoclonal antibody (clone 7H8.2C12, Pharmingen) and a polyclonal rabbit anti-serum (Susin et al. 1999) respectively.

Swelling of isolated mitochondria

Table F1:

Tox0, Tox1, Tox5, Tox6 induce permeability transition pore (PTP) openning

| Name of molecules 5 μM | Induction of Mitochondrial swelling (sw) +++ rapid sw; ++ low sw; + very low sw; - no sw t 20 min |
|------------------------|---|
| - | - |
| Tox0 | +++ |
| Tox1 | ++ |
| Ctr1Tox1 | - |
| Ctr2Tox1 | - |
| Ctr3Tox1 | + |
| Ctr4Tox1 | - |
| Tox5 | ++ |
| Tox6 | ++ |

Table F2: Save 1 and Save2 inhibit atractyloside-induced PTP openning

| Name of molecules | Mitrochondrial swelling (sw) | |
|--|------------------------------|--|
| | % | |
| ** | 2 | |
| Ca 2+ 100µM | 100 | |
| Atractyloside 600 µM | 110 | |
| Save I 5 μ M | 2 | |
| Save I 5μ M + Atr 600μ M | 5 | |
| Save I 20 μ M | 12 | |
| Save I $20\mu M + Atr 600\mu M$ | 12 | |
| Save II 10µM | 2 | |
| Save II 20 μ M | 16 | |
| Save II $10\mu\text{M} + \text{Atr } 600\mu\text{M}$ | 16 | |
| Save II $20\mu M + Atr 600\mu M$ | 16 | |

G. ANT purification and reconstitution in liposomes

ANT was purified from rat heart mitochondria as previously described (8). After mechanical shearing, mitochondria were suspended in 220 mM mannitol, 70 mM sucrose, 10 mM Hepes, 200 µM EDTA, 100 mM DTT, 0.5 mg/ml subtilisin, pH7.4, kept 8 min on ice and sedimented twice by differential centrifugations (5 min, 500 x g, and 10 min, 10,000 x g). Mitochondrial proteins were solubilized by 6% [v:v] Triton X-100 (Boehringer Mannheim) in 40 mM K₂HPO₄, 40 mM KC1, 2 mM EDTA, pH 6.0, for 6 min at RT and solubilized proteins were recovered by ultracentrifugation (30 min, 24,000 x g, 4°C). Then, 2 ml of this Triton X-100 extract was applied to a column filled with 1 g of hydroxyapatite (BioGel HTP, BioRad), eluted with previous buffer and diluted [v:v] with 20 mM MES, 200 µM EDTA, 0.5% Triton X-100, pH6.0. Subsequently, the sample was separated with a Hitrap SP column using a FPLC system (Pharmacia) and a linear NaCl gradient (0-1M). Proteins concentration was determined using microBCA-assay (Pierce, Rockfoll, Illinois). Purified ANT and/or recombinant Bcl-2 were reconstituted in PC/cardiolipin liposomes. Briefly, to prepare liposomes, 45 mg PC and 1 mg cardiolipin were mixed in 1 ml chloroform, and the solvent was evaporated under nitrogen. Dry lipids were resuspended in 1 ml liposome buffer (125 mM sucrose + 10 mM -2hydroxyethylpiperazine-N'-2 ethanesulfonic acid; Hepes, pH 7.4) containing 0.3% n-octyl-\(\beta\)-Dpyranoside and mixed by continuous vortexing for 40 min at RT. ANT (0.1 mg/ml) or recombinant Bcl-2 (0.1 mg/ml) were then mixed with liposomes [v:v] and incubated for 20 min at RT. Proteoliposomes were finally dialysed overnight at 4°C.

H. Pore opening assay

ANT-proteoliposomes are sonicated in the presence of 1 mM 4-MUP and 10 mM KC1 (50W, 22sec, Branson sonifier 250) on ice as previously described (28). Then, liposomes were separated on Sepadex G-25 columns (PD-10, Pharmacia) from unencapsulated products. 25 µl-aliquots of liposomes were diluted to 3 ml in 10 mM Hepes, 125 mM saccharose, pH 7.4, mixed with various concentrations of the proapoptotic inducers and incubated for 1 h at RT. Potential inhibitors of mitochondrial membranes permeabilization such as BA, ATP and ADP, were added to the liposomes 30 min before treatment. After addition of 10 µl-alkaline phosphatase (5 U/ml, Boehringer Mannheim) diluted in liposomes buffer + 0.5 mM MgCl₂, samples were incubated

for 15 min at 37°C under agitation and the enzymatic conversion of 4-MUP in 4-MU was stopped by addition of 150 µl Stop buffer (10 mM Hepes-NaOH, 200 mM EDTA, pH 10). The 4-MU-dependent fluorescence (360/450 nm) was subsequently quantitated (28) using a Perkin Elmer spectrofluorimeter. Attractyloside, a pro-apoptotic permeability transition inducer, was used in each experiment as a standard to determine the 100% response. The percentage of 4-MUP release induced by Vpr-derived peptides or pTarg-ptox was calculated as following: [(fluorescence of liposomes treated by pTar-pTox - fluorescence of untreated liposomes) / (fluorescence of liposomes treated by attractyloside - fluorescence of untreated liposomes)] x 100.

ANT pore opening assay:

Table H1: examples of fuctionnal interaction between ANT and Tox or Save constructs.

Tox0 and Tox6 induce ANT-protéoliposomes permeabilisation. Save1 and Save2 block

Atractyloside (Atra) -induced ANT-protéoliposomes permeabilisation

| molecules | Permeabilisation of ANT - |
|------------------------------------|---------------------------------------|
| | proteoliposomes |
| | +++ high UMP release; ++ UMP release; |
| | + low UMP release; - no UMP release |
| - | - |
| Atra 50μM | + |
| Atra 100µM | ++ |
| Atra 200µM | +++ |
| Tox0 (Biotin-Vpr52-96) 2μM | +++ |
| Τοχ6 5μΜ | ++ |
| Biotin-Vpr71-96[C76S] 5μM | ++ |
| Savel 5µM | - |
| Atra 200μM + Save1 5μM | - |
| Save2 5µM | - |
| Atra 200 μ M + Save2 5 μ M | • |

I. Binding assays and western blot

Mouse liver mitochondria were isolated as described (zamzami *et al.*, 2000). For the determination of cytochrome C release, supernatants from pTarg-pTox treated mitochondria (6800 g for 15min; then 20 000 g for 1 h; 4°C) were frozen at -80°C until immunodetection of cytochrome c (mouse monoclonal antibody clone 7H8.2CI2, Pharmingen). For binding assays, purified mitochondria were incubated (250 μg of protein in 100 μl swelling buffer) for 30 min at

RT 5 μ M (binding assay) of pTarg-pTox or biotin-pTarg-pTox. Mitochondria were lysed either after incubation with biotinylated Vpr52-96 (upper panel) or lysed before (lower panel) with 150 μ l of a buffer containing 20 mM Tris/HCl, pH 7.6; 400 mM NaCl, 50 mM KCl, 1mM EDTA, 0.2 mM PMSF, aprotinin (100U/ml), 1% Triton X-100 and 20% glycerol. Such extracts were diluted with 2 volumes of PBS plus lmM EDTA before the addition of 150 μ l avidin-agarose (ImmunoPure, from Pierce) to capture the biotin-labeled Vpr52-96 complexed with its mitochondrial ligand(s) (2 hours at 4°C in a roller drum). The avidin-agarose was washed batchwise with PBS (5 x 5 ml; 1000 g, 5 min, 4°C), resuspended in 100 μ l of 2 fold concentrated Laemmli buffer containing 4% SDS and 5 mM β -mercaptoethanol, incubated 10 min at RT and centrifuged (1000 g, 10 min, 4°C). Finally, the supernatants were heated at 95°C for 5 min and analysed by SDS-PAGE (12%), followed by Western blot and immunodetection with a rabbit polyclonal anti-serum against human ANT (kindly provided by Dr. Heide H. Schmid; The Hormel Institute, University of Minnesota, MI; Ref).

J. Flow cytometric analysis of purified mitochondria

Mouse liver mitochondria are isolated as described (zamzami *et al.*, 2000). Purified mitochondria are resuspended in PT buffer (200 mM sucrose, 10 mM Tris-MOPS (pH 7.4), 5 mM Tris-succinate, 1 mM Tris-phosphate, 2 μ M rotenone, and 10 μ M EGTA). Cytofluorometric (FACSVantage, Beckton Dickinson) detection is restricted to mitochondria by gating on the FSC/SSC parameters and on the main peak of the FSC-W parameter. Confirmation *a posteriori* of the validity of these double gating is obtained by labeling of mitochondria with the $\Delta\Psi_m$ -insensitive mitochondrial dye MitoTracker® Green (75 nM; Molecular Probes; green fluorescence). To determine the percentage of mitochondria having a low $\Delta\Psi_m$, the $\Delta\Psi_m$ -sensitive fluorochrome JC-1 (200 nM; 570-595 nm) is added 10 min before CCCP or pTarg-pTox molecules. Percentage of mitochondria having a low $\Delta\Psi_m$, is determined in dot-plot FSC/FL-2 (red fluorescence) windows.

K. Cell-free system of apoptosis

AIF activity in the supernatant of mitochondria is tested on HeLa cell nuclei, as described (Susin *et al.*, 1997b). Briefly, AIF-containing supernatants of mitochondria are added to purified HeLa nuclei (90 min, 37°C), which are stained with propidium iodide (PI; 10 μg/ml; Sigma Chemical Co.) and analyzed in an Elite II cytofluorometer (Coulter) to determine the frequency

of hypoploid nuclei. In some experiments isolated mitochondria, cytosols from Jurkat or CEM cells (prepared as described (Susin *et al.*, 1997a)), and/or pTarg-pTox are added to the nuclei. Caspase activity in the mitochondrial supernatant was measured using Ac-DEVD-amido-4-trifluoromethylcoumarin (Bachem Bioscience, Inc.) as fluorogenic substrate.

L. Purification and reconstitution of PTPC in liposomes

PTPC from Wistar rat brains are purified and reconstituted in liposomes following published protocols (Brenner et al., 1998; Marzo et al., 1998b). Briefly, homogenized brains are subjected to the extraction of triton-soluble proteins, adsorption of proteins to a DE52 resin anion exchange column, elution on a KC1 gradient, and incorporation of fractions with maximum hexokinase activity into phosphatidy1choline/cholesterol (5: 1, w:w) vesicles by overnight dialysis. Recombinant human Bcl-2 (1-218) lacking the hydrophobic transmembrane domain (Δ 219-239), produced and purified as described (Schendel et al., 1997) are added during the dialysis step at a dose corresponding to 5% of the total PTPC proteins (approximately 10 ng Bcl-2 per mg lipids). Liposomes recovered from dialysis are ultrasonicated. (120 W) during 7 sec in 5 mM malate and 10 mM KCl, charged on a Sephadex G50 columns (Pharmacia), and eluted with 125 mM sucrose + 10 mM HEPES (pH 7.4). Aliquots (approx. 10⁷) of liposomes are incubated during 60 min at RT in 125 mM sucrose + 10 mM HEPES (pH 7.4) in the presence or absence of pTarg-pTox, [52-96]Vpr or atractyloside. Then, liposomes are equilibrated with 3,3'dihexylocarbocyanine iodide (DiOC₆(3), 80 nM, 20-30 min at RT; Molecular Probes), and analyzed in a FACS-Vantage cytofluorometer (Becton Dickinson, San José, CA, USA) for DiOC₆(3) retention, as described (Brenner et al., 1998; Marzo et al., 1998b).

Triplicates of 5 x 10^4 liposomes are analyzed and results are expressed as % of reduction of DiOC₆(3) fluorescence, considering the reduction obtained with 0.25% SDS (15 min, RT) in PTPC liposomes as 100% value.

Examples of specific peptides and constructs relating to this invention that can be utilized in carrying out the foregoing techniques are shown in Tables I, II, and III, as well as any chimeric molecule that is a combination between TARG and TOX or TARG and SAVE peptides or peptidomimetics.

EXAMPLE 6

Surface plasmon resonance indicates that Tox0, Tox1, Tox5, Tox6, Save1 binds purified ANT but not purified VDAC.

Methodology.

Sensor Chips SA (streptavidin coated sensor chips) were used for immobilisation of the different peptides. Tox1 was immobilised at a density of 0.7 ng/mm², Tox0 at a density of 3.7 ng/mm², Ctr1Tox0 at a density of 1.4 ng/mm², Tox5 at a density of 1 ng/mm². Tox6 at a density of 1 ng/mm2, Save1 at a density of 1.3 ng/mm², and the control peptide at a density of 0.8 ng/mm². Association and dissociation kinetics of ANT and VDAC interactions were followed at a rate of 10 $\mu L/min$ for 10 minutes (5 minutes association and 5 minutes dissociation). The ligand was regenerated with a 1 minute flux of KSCN 3M. The obtained sensorgrams were analysed by the BlAeval 3.1 software using the method of double references (Myszka D.G. 2000. Kinetic, equilibrium and thermodynamic analysis of macromolecular interactions with BIACORE. Methods Enzymol. 323:325-340). From the sensorgrams with the ligands were first substracted the sensorgrams obtained with the corresponding analyte solvents. A second substraction was performed with the sensorgrams obtained with the control peptide ligand. The control peptide for the Tox and Save peptides was biot-H19C corresponding to the sequence of the β2-adrenergic receptor (Lebesgue D., Wallukat G., Mijares A., Granier C., Argibay J., and Hoebeke J. (1998) An agonist-like monoclonal antibody to the human β 2-adrenergic receptor. Eur.J. Pharmacol. 348:123-133). The control peptide for Tox0 was Ctr1Tox0.

Results.

Figure 6 shows the interaction between ANT and Vpr for 4 ANT concentrations (6.25 to 50 nM). The sensorgrams were best analysed using the simple Lagmuir model with drifting baseline and resulted in a Kd of 0.15 nM with a Rmax of 160 ($_{\rm X}2 = 7.24$). The same analysis was performed for the sensorgrams showing the interaction between ANT and Tox1 (Figure 7). Studying the VDAC interaction both with Tox0 and Tox1 at VDAC concentrations which were ten times higher (Figure 8 and 9), the sensorgrams showed only extremely low association with

the peptide ligand and the obtained curves could not be analysed by the different Langmuir bindings models.

Thee other peptides were tested for their interaction with ANT at a concentration of 50 nM (Figure 10). Purified ANT recognised Tox5, Tox6, and Save1 with relative affinities of respectively 0.1, 0.7, and 0.01 nM. These value being obtained at only one ANT concentration only give the relative affinity of ANT for the three peptides. Again, the use of 50 nM VDAC to interact with the same peptides did not result in any specific binding as shown in Figure 11.

The following references have been cited herein. The entire disclosure of each reference cited herein is relied upon and incorporated by reference herein.

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What is claimed is:

1. Method for inducing or preventing the apoptosis of eukaryotic cells comprising the homing on specific tissue cell population of a chimeric bifunctional molecule able to modulate-the activity of permeability transition pore complex (PTPC).

- 2. A method according to claim 1, wherein said chimeric molecules modulate the activity of the permeability transition pore complex (PTPC) of a specific eukaryotic cell by the regulation of opening or the closing of said pore.
- 3. A method according to claim 1 or 2, wherein said chimeric molecules comprising at least a first functional molecule and a second functional molecule, wherein said first functional molecule has the function to target specifically a tissue cell population, and the second functional molecule has the function to regulate the apoptosis activity linked to the permeability transition pore complex (PTPC) of said specific calls.
- 4. A method according to claim 3, wherein said chimeric molecules comprising at least a first functional molecule and a second functional molecule, wherein said first functional molecule has the function to target and to enter specifically in a tissue cell population and the second functional molecule has the function to regulate the apoptosis activity linked to the permeability transition pore complex (PTPC) of said specific cells.
- 5. A method according to claim 3, wherein said chimeric molecules comprising at least a first functional molecule and a second functional molecule, wherein said first functional molecule has the function to target and to enter specifically in a tissue cell population of interest and the second functional molecule has the function to target specifically and inducing or preventing the death of said cells by apoptosis by the regulation of the

opening or the closing of the permeability transition pore complex (PTPC) of mitochondria or a fragment thereof.

6. A method according to claim 4, wherein said chimeric molecule has the formula:

Targ-Tox,

wherein Tox is a viral or a retroviral apoptotic peptide or a peptidomimetic or a fragment of a protein that interacts with permeability transition pore complex (PTPC) of a specific eukaryotic cell to cause apoptosis of the cell; and Targ is chosen from:

an antibody,

an antibody fragment,

arecombinant antibody fragment,

M350/ScFv,

V461/ScFv,

a homing peptide, and

any peptide chosen in Table III,

wherein said molecule binds and enters the cell specifically.

7. A method according to claim 5, wherein said chimeric molecule has the formula

Targ-Save,

wherein Save is a viral or a retroviral or a cellular antiapoptotic peptide or peptidomimetic or a fragment of protein that interacts with permeability transition pore complex (PTPC) of a specific eukaryotic cell to prevent the apoptosis of the cell with the proviso that when Save peptide is a viral peptide, Save is not vMIA protein of Cytomegalovirus; and

Targ is chosen from:

an antibody,

an antibody fragment,

a recombinant antibody fragment,

M350/ScFv,

a homing peptide, and

any peptide chosen in Table III,

wherein said molecule binds and enters the cell specifically.

- 8. A method according to anyone of claims 1 to 7, wherein said chimeric molecules comprises a Mitochondrial Localisation Sequence (MLS), which has the function to address specifically the second functional molecule to mitochondrial or intermembrane space-of the mitochondria.
- 9. A method according to claims 1, 2, 3, 4, 5, 6 and 8, wherein Tox is chosen from the group of peptides of Table I.
- 10. A method according to claims 1, 2, 3, 4, 5, and 7, wherein Save is chosen from the group of peptides of Table II.
- 11. A method according to any one of claims 1 to 10, wherein the second functional molecule of said chimeric molecules has the function to interact specifically with ANT of the PTPC of mitochondria also refers to as adenine nucleotide translocator isoforms 1, 2, or 3.
- 12. A chimeric bifunctional molecule capable to enter specifically in a tissue cell population for induce or prevent death of said cell by apoptosis and comprising at least a first functional molecule covalently linked to a second functional molecule, wherein said first

functional molecule has the function to target and to enter specifically in a tissue cell population of interest and the second functional molecule has the function to target specifically and inducing or preventing the death of said cells by apoptosis by the regulation of the opening or the closing of the permeability transition pore complex (PTPC) of mitochondria or a fragment thereof.

13. A chimeric molecule according to claim 12 which has the formula:

Targ-Tox,

wherein Tox is a viral or a retroviral apoptotic peptide or peptidomimetic or a fragment of a protein that interacts with PermeabilityTransifion Pore Complex (PTPC) of a specific eukaryofic cell to cause apoptosis of the cell; and

Targ is chosen from:

an antibody,

an antibody fragment,

a recombinant antibody fragment,

M350/ScFv,

V461/ScFv,

a homing peptide, and

any peptide of Table III,

wherein said molecule binds and enters the cell specifically.

14. A chimeric molecule according to claim 12 which has of the formula

Targ-Save

Wherein Save is a viral or a retroviral or a cellular antiapoptotic peptide or peptidomimetic or a fragment of protein that interacts with Permeability Transition.

Pore Complex (PTPC) of a specific eukaryotic cell to prevent apoptosis of the cell, with the proviso that when Save peptide is a viral peptide, Save is not vMIA protein of Cytomegalovirus;

and Targ is chosen from:

an antibody,

an antibody fragment,

a recombinant antibody fragment,

M350/ScFv,

a homing peptide, and

any peptide of Table III,

wherein said molecule binds and enters the cell specifically.

- 15. A chimeric molecule according to any of claims 12 to 14 comprising a mitochondrial localisation sequence (MLS) which has the function to address specifically the second functional molecule to mitochondrial membranes or intermembrane space.
- 16. A chimeric molecule according to claims 13 or 15, wherein Tox is chosen from the group of peptides of Table I.
- 17. A chimeric molecule according to claims 14 and 15, wherein wherein Save is chosen from the group of peptides of Table II.
- 18. A chimeric molecule according to claims 13, 15 and 16, wherein the Targ and Tox peptides are covalently bonded through a peptide linker comprising 3 to 18 amino acids.

19. A chimeric molecule according to claims 14, 15 and 17, wherein the Targ and Save peptides are covalently bonded through a peptide linker comprising 3 to 18 amino acids.

- 20. A vector encoding a chimeric molecule as claimed in any one of claims 12 to 19.
- 21. A hybridoma secreting Targ according to claim 13 or 14 and deposited at the National Collection of Culture and Microorganism (C.N.C.M.) on January 24, 2001, under the accession number n° I 2617.
- 22. A purified monoclonal antibody encoded by the hybridoma of claim 21.
- 23. A recombinant host cell comprising a vector as claimed in claim 20.
- 24. A cancer cell having a tumor associated antigen on the surface thereof to which is bound the chimeric molecule as claimed in any one of claims 12 to 19.
- 25. A method of determining the presence of a cancer cell having a tumor-associated antigen on the surface thereof in a biological sample comprising:
 - a) contacting a biological sample of interest with a chimeric peptide molecule according to claims 12 to 19 under conditions to permit the binding between the chimeric peptide according to the invention and the antigen on the surface of the. cancer cell.
 - b) detecting the binding by usual technique; and
 - c) optionally quantifying the binding detected in step b).
- 26. A method for inducing death by apoptosis in a tumoral or viral infected cell having a tumor-associated antigen on surface thereof in a biological sample comprising: contacting a biological sample of interest with a chimeric peptide molecule according to claims 16 or 17 under conditions to permit the binding between the chimeric peptide

according to the invention and the antigen on the surface of the cancer cell and for a time sufficient to allow the entry inside the cell and death cell by apoptosis or viral infected cells.

- 27. A method for prevent cell death by mitochondrial apoptosis comprising contacting a biological sample of interest with a chimeric molecule, **molecule** according to claims 17 or 19 under conditions to permit the binding between the chimeric molecule according to the invention and the cell of interest and for a time sufficient to allow the entry inside cell of interest and prevent the cell death by apoptosis.
- 28. A method for prevent cell death according to claim 27, wherein the cells of interest are choosen among the following cell populations: neurons, cardiocytes, and hepatocytes.
- 29. A method for identifying an active agent of interest that interacts with the activity of the permeability transition pore complex (PTPC) comprising
 - a) contacting a biological sample containing cells with permeability transition pore complex (PTPC) with a chimeric peptide according to claims 12 to 19 in the presence of a candidate agent; and
 - b) comparing the binding of the chimeric peptide with the permeability transition pore complex (PTPC) in absence of said agent.
 - c) optionally, testing the activity of said selected agent on a preparation of a cellular extract comprising subcellular elements with the permeability transition pore complex (PTPC).

30. A method for identifying an active agent of interest that interacts with ANT peptide of permeability transition pore complex (PTPC) comprising:

- d) contacting a biological sample containing cells with ANT peptide of permeability transition pore complex (PTPC) with a chimeric peptide according to claims 12 to 19 in the presence of a candidate agent; and
- e) comparing the binding of the chimeric peptide with the ANT peptide of the permeability transition pore complex (PTPC) in absence of said agent.
- f) optionally, testing the activity of said selected agent on a preparation of a cellular extract comprising subcellular elements with the ANT peptide of the permeability transition pore complex (PTPC).
- 31. A method of identification of mitochondrial antigen, said antigen having the capacity to interact with a macromolecule or a molecule or a peptide carrying the characteristic of Tox according to claims 13 or 16.
- 32. A method of identification of mitochondrial antigen, said antigen having the capacity to interact with a macromolecule or a molecule or a peptide carrying the characteristic of save according to claims 14 or 17.
- 33. A method of treatment or of prevention of a pathological infection or disease comprising the administration to a patient of the pharmaceutical composition containing at least a chimeric molecule according to any of claims 12 to 19.
- 34. A pharmaceutical composition comprising at least a chimeric molecule according to claims any of 12 to 19.

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| cur che din ten seb diu | ser OPA | ars ala | OFA pro ala | 33ء | leu | | pro | arc | ser | ala | cys pro | OPA | up | gla | arg | C)A | val | gln | œ; |
| 10:1/10: | | | 1111/371 | | | | | | | | 1141/18 | 1 | | | | | | | |
| ACT CTT GTT TTT TAA CAA | UP' DED | CCC TIT | TIT CC CC | CCY | ಯ | C.L.L. | \propto | ಯಾ | cc: | <u>c</u> | ici cir | ಯ | TGA | AIC | TCS | CYY | TCA | حث | TAG |
| thr leu val phe CCH gln 1171/391 | Va. p. 0 | عاام و عد | 1201/401 | pro | pro. | reu | ara | aia | erg | | | | CPA | ne C | ser | şin | 5 C | ala | AMB |
| דכא ככא אכד קדד דככ דכד | ಯ ಯ | כככ פודו | כדד דכא דכם | ಂದರ | GAT | car | ACT | TCC | csc | 777 | 1231/41 AGA GCA | ٠ | GAG | GAA | TTA | ىت | ٠ | لابت | 444 |
| ser bro cur var che ser | bio bio | pro val | val OPA ser | | چې | وجه | t!= | CYS | arg | cys | arg ala | leu | glu | glu | leu | leu | leu | leu | lvs |
| | | | 1737/471 | | | | | | | | 1171/20 | | | | | | | | |
| CCC ATT CTT GTA ATT CTA | 100 007 | AAG GCA | ATT TGG ACT | . 15, | 722 | TCA | сć. | CÀA | TEA | ೧୯୯୯ | CGC ATT | TAG | TAA | TG4 | CCA | CTC. | TAT | ಯಾ | CCT |
| ala ile leu val ile leu 1351/451 | وحت لاحت | TAP CIT | 1381/461 | ser | CC | ser | ala | Gin | ser | arg | arg ile | , AVE | OCH | CPA | . دلد | leu | ty | ala | ala |
| GCA AAT ACA GCG GGT CGC | בבב בבב | TCA CGA | CGC TGT TAG | AGG | TAC | ಹಾ | ccc | CAT | 1-1 | CC.A | 1411/47: TGG TCT | CCT | CAA | ATA | نجمد | ، بالمثلا | | 2777 | ተኔተ |
| are ear, ere dry ard | entq crq | ser arg | בדק כעים אינם | ٠., | AM3 | Sly | פזק | his | phe | G1Y | tro ser | ala | gin | ile | בליב | ile | .v. | ile | EVE |
| 74-71-407 | | | 14/1/491 | | | | | | | | さらのこ ノミカリ | | | | | | | | |
| TOT CTA CAT GAA CAC GTA | TAG CTT | TAT CAC | AAA CTS TAT | YII | Ξ.T.Y | AAC | TCT | TAG. | CGY | ca: | CCI 1333 | CCY | CGA | YCC . | CCY | ccr . | CL. | GGT | œ |
| cys leu his glu his val 1531/511 | ryss ten | tyt ms | 1ys 1eu tyr 1561/521 | ile | l eu | കാ | che | AMB. | | ೭೯ | bro mb | ಶಿಬಾ | متد | באב | gly | pro. | val. | 2,7 A | grç |
| GCT CTA GCA CGT ACC GCA | SCT TGA | ACG TAT | CTT CTC CAA | ATT | TAA | ستد | ٠ | ראס. | . بلملمة | TA A | 1591/531 | CC3 | بتعيث | TT:a | ~a~ | »~~ · | ا تعاد | | ~~~ |
| are ses are are the are | gly OPA | thr tyr | leu leu gin | 110 | OC: | ile | leu | cln : | ohe : | co: | are elu | 020 | Dhe | OPA | tvr | thr | | י שנט | 0~. |
| 1021/341 | | | 1551/551 | | | | | | | | 1681/561 | | | | | | | | |
| TTT GCA ACA ACT ATT GTT | TTT TAX | ووود نهمه | CLY YYC LLY | Lic | ∞ | TAA | CZλ. | ATA | ATT. | AAA ' | TAT GGG | ಯಸಿ | ACA | TGC | GCC | ccr . | ACA . | אכא ו | CIC |
| phe ala thr thr ile val 1711/571 | prie our | ard TAR | 104 AST. 104 | leu | crp | CC | مام | ile | ile | lys | tyr gly | aīx | thr | c\2 | ala | ala | - | בלים | leu |
| GTC GTT ATG AAC GCA GAC | eee eee | פסד כדים | 1741/551 GGC GCA AGC | GC: | 722 | AAC - | ، نملک | ، تلم | | . سبب | 1771/591 Caa Car | 22 | 272 | רבד י | ~:- | 222 | . مے | | - |
| val val met asn ala asp | gly ala | gly leu | gly ala ser | 517 | CCH | 280 | val | ieu . | · | val | oln arm | alv | lvs | his | - LOC . | han i | | -1 - - | 1747 |
| 2001, | | | 1221/217 | | | | | | | | 1861/671 | | | | | | | | |
| TAC AGT TIT GAT TIG CAT | ATT AAC | GGC GAT | TEL TER ART | | CLI | ATT. | AAT | TAA . | ATA (| GTT. | ATG ACG | CCT . | ACA . | ACT (| ¢¢¢ (| csc : | CCC (| TT. | TGλ |
| tyr ser phe asp leu his 1891/631 | ile asn | aly was | phe leu asa 1921/641 | بكت | leu | ile | с н (| CCH | iie ' | val : | et thr | ĎΣΟ | C | 672 1 | pro . | ary 1 | وعو | rta (| AGC |
| | ، عندن منتن | TOA COO | 1251/041 | شعمك | | ~~` | | ~~~ | ~. | ; | 1951/651 | · ~~ | m | ~ ., | | ~~ . | | | |
| | | | | 101 | clv | 2 | ~~~ ! ~ ~ ~ . | | | 200 | alv alv | 270 | DD7 | חדח : | - 1 - 1 | : ناباد د ماد | rsc (| .GC / | منته |
| כדכ פכד ככג ככד כבג פכג | val ara | OPA are | leu pro pro | | | _ 9 | | G-9 (| _ , | 010 | 3+1 3-1 | | | P 1 | | | | | -11 |
| CTC GCT SCA CCT CEA GCA leu ala ala pro arg ala 1981/561 | Agt gid | OPA erg | 2011/671 | | | | | | | | 2041/681 | | | | | | | | |
| CTC GCT SCA CCT CEA GCA leu ala ala pro arg ala 1981/561 CGA CCC ACA AGT ATC TGT | ACA CCG | OPA EIG AAT GAT | 2011/671 COT CGG GCC | AAG | GC3 | المث | csc (| ، جب | ~a : | ا ست | 2041/681 33C AAT | Tank | - | | ر للما | 72.2 2 | | · . | EAC |
| CTC GCT GCA CCT CEA GCA leu ala ala pro arg ala 1981/561 CGA CGC ACA AGT ATC TGT arg arg thr ser ile cys | ACA CCG | OPA EIG AAT GAT | red pro pro 2011/671 COT CGG GCC arg arg ala | AAG | GC3 | المث | ccc (| ، جب | ~a : | ا ست | ~~ AAT | Tank | - | | ر للما | 72.2 2 | | · . | EAC LYT |
| CTC GCT GCA CCT CEA GCA leu ala ala pro arg ala 1991/561 CGA CGC ACA AGT ATT TGT arg arg thr ser ile cys 2071/691 | ACA CCC . | AAT GAT asn asp | red pro pro 2011/671 COT CGG GCC arg arg ala 2101/701 | aac iys | GCA ala | جيم دويد ، | arg ; | cci (| CCA I | ACT (| GGC AAT | ATT (| GGC . | AAA : | PTC (| SAA ; | AT / | TA 1 | tyr |
| CTC GCT GCA CCT CEA GCA leu ala ala pro arg ala 1981/561 CGA CGC ACA AGT ATC TGT arg arg thr ser ile cys 2071/691 AGT TGG GTT GTT TGC GCA | ACA CCC . TAT CTA | AAT GAT asn asp | 2011/671 COT CGG GCC arg arg ale 2101/701 COT TGG GCA | AAG lys TGT | GCA ala | سر جیم دین | ar g ; | cci (| OCA 2 pro : | ACT (| GGC AAT gly asn 2131/711 | ATT (| ecc. | AAA : | phe o | SAA ; Slu ; | SAT / | ile (| tyr |
| CTC GCT GCA CCT CEA GCA leu ala ala pro arg ala 1981/561 CGA CGC ACA AGT ATC TGT arg arg thr ser ile cys 2071/691 AGT TGG GTT GTT TGC GCA ser trp val val cys ala 2151/721 | ACA CCC . TAT CTA . tyr leu | AAT GAT AST OSP TOO TOO SET TIP | leu pro pro 2011/671 COT CGG GCX arg arg ale 2101/701 COT TGG GCA arg trp ala 2191/731 | che Loi Ihe Yac | GCA ala ACC thr | TCC | GAA (Glu | oci (cci ; pro ; | DCA P pro : TGA P DPA ; | Ser : | GGC AAT gly asn 2131/711 GCA TGC ala cys | ATT (ile ; AAG (lys ; | GGC . Gly GCG . | AAA : AAA : AAA : | PTC (phe s PTA : | CAA ; plu d CAAT (| AT : | ile (| eyt Bat Bep |
| CTC GCT GCA CCT CEA GCA leu ala ala pro arg ala 1991/561 CGA CGC ACA AGT ATT TGT arg arg thr ser ile cys 2071/691 AGT TGG GTT GTT TGC GCA ser trp val val cys ala 2151/721 TAG TGC GAT TAA AAC GTT | ACA CCC . TAT CTA tyr leu | AAT GAT asn asp TCG TGG ser trp | 2011/671 COT CGG GCC arg arg ale 2101/701 COT TGG GCA arg trp ale 2191/721 TTT TAA TCA | בכב בלב בלב אצנ אצנ אצנ | GCA ala ACC thr | COT ALTY Ser | GAA (Glu (| era (cci (| DCA A | AGT (ser : | SSC AAT gly asn 2131/711 SCA TGC ala cys 2221/741 | ATT dile | sec . gly ccg . pro | AAA : | PTC (phe q PTA (leu (| SAA ; glu d AAT (| AAT A | ile (| eyt Eat Esp |
| CTC GCT GCA CCT CEA GCA leu ala ala pro arg ala 1981/561 CGA CGC ACA AGT ATT TGT arg arg thr ser ile cys 2071/691 AGT TGG GTT GTT TGC GCA ser trp val val cys ala 2151/721 TAG TGC GAT TAA AAC GTT AME Cys asp CCH asp val | ACA CCC . TAT CTA tyr leu | AAT GAT asn asp TCG TGG ser trp | 2011/671 2011/671 2017/606 GCX arg arg ale 2101/701 CUT TGG GCX arg trp ala 2191/731 TTT TAA TCA phe CCH ser | בכב בלב בלב אצנ אצנ אצנ | GCA ala ACC thr | COT ALTY Ser | GAA (Glu (| era (cci (| DCA A | AGT (ser : | SSC AAT gly asn 2131/711 SCA TGC ala cys 2221/741 | ATT dile | sec . gly ccg . pro | AAA : | PTC (phe q PTA (leu (| SAA ; glu d AAT (| AAT A | ile (| eyt Eat Esp |
| CTC GCT GCA CCT CEA GCA leu ala ala pro arg ala 1981/561 CGA CGC ACA AGT ATC TGT arg arg thr ser ile cys 2071/691 AGT TGG GGT GTT TGC GCA ser trp val vel cys ala 2161/721 TAG TGC GAT TAA AAC GTT AME CYS asp CCH asm val 2251/751 | ACA CCC : the pro that CTA : tyr leu GTA CAT : val his | AAT GAT AAT GAT ESD TOO TOO TOO SET LTP COT COC Pro ATG | 2011/671 COT CGG GCC arg arg ale 2101/701 COT TGG GCA arg trp ala 2191/721 TTT TAA TCA phe CCH ser 2281/761 | 5.2 25.2 5.2 15.1 13.2 73.2 | GCA ala ACC thr CGT | CGT TCC Ser CGA | GAA (GAA (Glu (TTA (leu (| CCT (pro ; ccT ' ary (AAT (asn (| DCA A | AGT (ser : TTT (phe : CCA : | SSC AAT gly asn 2131/711 SCA TGC ala cys 2221/741 ATC SAG ale glu | ATT (ile ; AAG (lys ; TCA ; ser ; | GGC . gly CCG . pro AGT (| AAA 1 AAA 1 AAA 1 BAT 0 | TTC (phe state of the state of | CAA (Called AAT (C | AAT / SEN : CAT : Dis : | ile (| eyt eat esp eaa eaa |
| CTC GCT GCA CCT CEA GCA leu ala ala pro arg ala 1981/561 CGA CGC ACA AGT ATC TGT arg arg thr ser ile cys 2071/691 AGT TGG GTT GTT TGC GCA ser trp val val cys ala 2151/721 TAG TGC GAT TAA AAC GTT AME CYS ass CCH ass val 2251/751 TGT TTT CTT TGT ATT CCC | ACA CCG thr pro TAT CTA tyr leu GTA CAT val his | AAT GAT AAT GAT AAT GAT TOO TOO SET LTP COT COC Pro ATG ACC GCA | 2011/671 COT CGG GCC arg arg ale 2101/701 COT TGG GCA arg trp ala 2191/731 TTT TAA TCA phe OCH ser 2281/761 GCG GCT ATT | AAG TOT TOT TOT TOT TOT | GCA ala ACD thr CCT arg | CGT ATF TCC Ser CGA AIC | GAA (glu (TTA (| CCT (pro ; ccT (pro ; hry (hry (hr) (| DCA A | AGT (ser : TTT (phe DCA ; | GGC AAT gly asn 2131/711 GCA TGC ala cys 2221/741 ATC GAG ile glu 2311/771 | ATT (ile ; AAG (lys ; TCA ; ser ; | SGC . Ply Pro AGT (| AAA : | TTC (phe quality of the phase | SAA ; Elu d AAT (Ean i ACT (| TAS TAS TAS TAS TAS TAS TAS TAS TAS | ile (| TAA TAA TAA |
| CTC GCT GCA CCT CEA GCA leu ala ala pro arg ala 1981/561 CGA CGC ACA AGT ATC TGT arg arg thr ser ile cys 2071/691 AGT TGG GGT GTT TGC GCA ser trp val vel cys ala 2161/721 TAG TGC GAT TAA AAC GTT AME CYS asp CCH asm val 2251/751 | ACA CCG thr pro TAT CTA tyr leu GTA CAT val his | AAT GAT AAT GAT AAT GAT TOO TOO SET LTP COT COC Pro ATG ACC GCA | Teu pro pro 2011/671 COT CGG CCC arg arg ala 2101/701 CUT TGG GCA arg trp ala 2191/711 TTT TAA TCA phe CCH ser 2281/761 GCG CGT ATT ala arg ile | AAG TOT TOT TOT TOT TOT | GCA ala ACD thr CCT arg | CGT ATF TCC Ser CGA AIC | GAA (glu (TTA (| CCT (pro ; ccT (pro ; hry (hry (hr) (| DCA A | AGT (ser : TTT (phe CA / Lla : TTT (TTT (| SSC AAT cly asn 2131/711 SCA TGC ala cys 2221/741 ATC GAG ile glu 2311/771 AAG TTA lys leu | ATT (ile ; AAG (lys ; TCA ; ser ; GTT ; | SGC . Ply Pro AGT (| AAA : | TTC (phe quality of the phase | SAA ; Elu d AAT (Ean i ACT (| TAS TAS TAS TAS TAS TAS TAS TAS TAS | ile (| TAA TAA TAA |
| CTC GCT GCA CCT CEA GCA leu ala ala pro arg ala 1981/561 CGA CGC ACA AGT ATC TGT arg arg thr ser ile cys 2071/691 AGT TGG GTT GTT TGC GCA ser trp val val cys ala 2151/721 TAG TGC GAT TAA AAC GTT AMB CYS asp CCH asn val 2251/751 TGT TTT CTT TGT ATT CCC cys phe leu cys ile pro | ACA CCC TAT CTA TYT leu GTA CAT Val his GAG TCA Glu ser | AAT GAT GAT SEN SEN TOO TOO TOO TOO TOO TOO TOO TOO TOO TO | 2011/671 COT CGG GCC arg arg ale 2101/701 COT TGG GCA arg trp ala 2191/731 TIT TAA TCA phe CCH ser 2281/761 GCG CGT ATT ala arg ile 2371/791 TTA ACA ATG | 250 252 252 253 254 254 255 255 255 255 255 255 255 255 | GCA ala ACC thr CCT arg ACA thr | COT ETT Ser CGA arg AAC asn | GAA (| CCT (pro pro pro pro pro pro pro pro pro pro | CCA Pro : TGA ' TGA ' TGA ' TGA ' TGC (TG | ACT (ser : TTT (phe : CCA : tota : Tys : | SSC AAT Gly asn 2131/711 SCA TGC allo Cys ATC GAG ile glu 2311/771 AAG TTA lys leu 2401/801 | ATT ile : AAG Iys TCA : Ser : val : | GGC . G1Y . CCG . PTO . AGT (SCI . SCI . SCI . | AAA : lys ; AAA : lys ; SAT : sasp ; phe : | TTC (phe property) | CAA ACT O | AAT STAN STAN STAN STAN STAN STAN STAN S | ile (| EXT EXT EXT EXT EXT EXT |

Fig 1B pACgp67-ScFv461 -> 1-phase Translation 16/01/1 22:01:48 Page 2 2431/811 2491/831 2461/521 ANT TAX ATA GCT TOC GAC GCA ACG TGC ACG ACG TGT GCA CGC GTT CCG GCA CGA CGA GCT TTG ATT GTA ATA AGT TTT TAC GAA GCG ATG ACA ash OCH ile ala cys asp ala the cys the ile cys ala arg val pro ala arg ala lee ile val ile ser phe tyr glu ala met the 2551/851 2521/841 2581/861 TEA COO COO TAG TEA CAA CEA TCA CEC CCA AAA GAA CTG CCG ACT ACA AAA TTA CCG ACT ATG TCG GTG ACG TTA AAA CTA TTA AGC CAC OPA pro pro AME OFA gln arg ser arg pro lys gln leu pro thr thr lys leu pro ser met ser vel thr leu lys leu leu ser his 2511/871 2541/881 CCA AND GAD COT TAG TOG AAT CAG GAD COD TOG TOG GAG AAG COD CGA AGT ATG GCG AAT GCA TOG TAT AAD GTG TOG AGT CCG CTC ATT pro ile asp ang AMS ser asn gln asp ang trp cys glu lys pro ang ser met ala asn ala ser tyn asn val trp ser pro leu ile 2701/901 2731/911 2761/921 AGA GCG TCA TGT TTA GAC AAG AAA GCT ACA TAT TTA ATT GAT CCC GAT GAT TTT ATT GAT AAA TTG ACC CTA ACT CCA TAC ACG GTA TTC arg ala ser cys leu asp lys lys ala thr tyr leu ile asp pro asp asp phe ile asp lys leu thr leu thr pro tyr thr val phe 2791/931 2851/951 TAC AAT GGC GGG GTT TTG GTC AAA AUT TCC GGA CTG CGA TTG TAC ATG CTG TTA ACG GCT CCC ACT ATT AAT GAA AUT ACC ACT tyr asn gly gly val leu val lys ile ser gly leu arg leu tyr met leu leu thr ala pro pro thr ile asn glu ile lys asn ser 2911/971 7881/961 2941/991 AAT TIT AAA AAA CGC AGC AAG AGA AAC ATT TGT ATG AAA GAA TGC GTA GAA GGA AAG AAX AAT GTC GTC GAC ATG CTG AAC AAC AAC ATT ash phe lys lys arg ser lys arg ash ile cys met lys plu cys val glu gly lys lys ash val val ash met leu ash ash lys ile 3001/1001 3031/1011 2971/991 ANT ATO CCT CCG TGT ATA AAA AAA AMA TTG AAC GAT TTG AAA GAA AAC AAT GTA CCG CCC GGC GGT ATG TAC ACG AAG AGG TTT ATA CTA ash met pro pro cys ile lys lys ile leu ash asp leu lys glu ash ash val pro arg gly gly met tyr arg lys arg phe ile leu 3091/1031 3061/1021 3121/1041 AND TITT THE ATT SEA AND STIG STT TES TOT SEE AND TOT SEA AND CON TOT TIN ATC AND SET CITS ACS CAT THE TAC AND CAD CAD TEE ash cys tyr ile ala ash val val ser cys ala lys cys glu ash arg cys leu ile lys ala leu thr his phe tyr ash his asp ser 3181/1061 3151/1051 3211/1071 AND TOTH OTH GOT GAN GIVE AND CATH CITH THE AND THAN THE CAN GATH OTH THAN AND THAN CON THE THE THE CAN AND AND AND THE GIVE GAN AND THE CONTROL G lys cys val gly glu val met his leu leu ile lys ser glm asp val tyr lys pro pro asn cys glm lys met lys thr val asp lys 3241/1081 3241/1081 3301/1101 CTC TOT CCC TOT GCT GCC AAC TOC AAG GGT CTC AAT CCT ACT TOT AAT TAT TGA ATA ATA ATA AAA CAA TTA TAA ATG CTA AAT TTG TTT TTT leu cys pro phe ala gly asn cys lys gly leu asn pro ile cys asn tyr OPA ile ile lys gln leu OCH met leu asn leu phe phe 3331/1111 3361/1121 3391/1131 ATT AAC GAT ACG AAC CAA ACG CAA CAA GAA CAT TITE TAG TAC TAT CTA TAA TTG AAA ACG CGT AGT TAT AAT CCC TGA GGT AAT ATT TAA ile asm asp thr asm glm thr glm glm bis leu AMB tyr tyr leu CCH leu lys thr azg ser tyr asm arg CPA gly asm ile CCH 3451/1151 3481/1151 ANT CAT TIT CAA ATG ATT CAC AGT TAA TIT GCG ACA ATA TRA TIT TAT TIT CAC ATA AAC TAG AGG CCT TGT GGT CTT CTT GGT AGT asn his the gln met lie his ser OCH phe ala thr ile OCE phe tyr phe his ile asm AMB thr pro cys arg leu leu arg ile 3511/1171 3541/1161 3571/1191 CCT TOT CIT ITT CAT TIT TOT COT CAT AAA AAT TAA CAT AGT TAT TAT COT ATC CAT ATA TGT ATC TAT CGT ATA CAG TAA ATT TIT TGT pro ser leu phe his phe ser pro his lys asn CCH his ser tyr tyr arg ile his ile cys ile tyr arg ile glu CCH ile phe cys 3601/1201 3631/1211 3651/1221 OUT CIT COG ACT CIG CIT TAA TEA TEA AAT TEA TAI AAT AAS CAA TEA AIT TOO CAT CCT COG TIT TOT ACA ATA TOT TOO COG CAT ACT val leu arg ser val leu leu OCH leu leu asn leu tyr asn gln OPA ile trp asp arg arg phe cys thr ile cys cys arg his ser 3781/1251 3311/1271 3841/1281 ACC CAG CTT CTT CTA CTT CAA TTA CAC CAT TIT TEA GCA GCA CCC GAT TAA CAT AAC TIT CCA AAA TGT TGT ACC AAC CGT TAA ACA AAA thr Gin leu leu leu val Gin leu his his phe leu ala ala pro asp CCH his asn phe pro lys cya tru asn arg CCH thr lys 3871/1291 3901/1301 3901/1301 3871/1291 ACA STE CAS STE COT THE STA SAS SAS STE STE STE STE STE STE STE STA AAA SAA SAA CAG SCA TIG SAA SGA GAC GAA ACT AAT thr val his leu pro the leu tyr tyr cys leu arg ala val val cys cys OCH lys CCH gln pro leu OCH OPA asp ala gln thr asn 3961/1321 4021/1341 ate ara and too and tot eta tea act and tat agt toe toa tat eat goa gat and tan and gat and eat etc oca and ana tan gir tite ile the asm trp lys cys leu ser ile tyr ser cys 02% tyr his gly asp asm OCH asm asp asm his leu ala asm lys OCH wal phe 4051/1351

TAC TOT TIT COT AAC AST TIT GIA ATA AAA ACT ATA AAT ATT CCG GAT TAT TAC COT CCC ACC ATT GGG CGG GGA TET ATG CTA CYT Cys phe arg asm ser phe wal ile lys lys pro ile asm ile pro asp tyr ser tyr arg pro thr ile gly arg gly ser mat leu 4141/1381

4171/1391

4201/1401 CTA GTA AAT CAG TCA CAC CAA GGC TTC AAT AAG GAA CAC ACA AGC AAG ATG GTA AGC GCT ATT GTT TTA TAT GTG CTF TTG GGG GGG GGG leu val asn gin ser his gin gly phe asn lys glu his the ser lys met val ser ale ile val leu tyr val leu leu ale ale ale 4231/1411

A 2561/1421 Tag HS Factor A 4291/1431 > SCF x 464 a VH

GCG CAT TOT GCC TIT GCC GAT CIT ggs tee CAT CAT CAC CAC CAC CAC att ges ggs aga GAA TIT CAG GTG CAG GTG AAG GAG TCA
ale his ser ale phe alevale asp leu gly ser his his his his his his ile glu gly att gglu phe gln val gln leu lys glu ser 4351/1451 4381/1461 GGA CCT GGC CTG GTG GCC CCC TCA CAG AGC CTG TCC ATC ACA TCC ACT GTC TCA GCG TTC TCA TTA ACC ACC TAT GGT GTA ACC TCG GTT gly pro gly leu val ala pro ser gln ser leu ser ile thr cys thr val ser gly phe ser leu thr ser tyr gly val ser trp val 4411/1471 4441/1481 4471/1491 COC CAS CCT CCA GGA AAG GGT CTG GAG TGG CTG GGA GTA ATA TGG GGT GAC GGG AGC ACA AAT TAT CAT TCA GCT CTC ATA TCC AGA CTG ary gin pro pro gly lys gly leu glu trp leu gly val ile trp gly asp gly ser thr asn tyr his ser ala leu ile ser are leu 4501/1501 4531/1511 4561/1521 AGO ATO AGO AAG GAT AAC TOO AAG AGO CAA GIT TIC ITA AAA CIG AAC AGT CIG CAA ACT GAT GAC ACA GCC ACG TAC TAC TGT GCC AAA ser ile ser lys asp asn ser lys ser gin val phe leu lys leu asn ser leu gin the asp asp thr ala thr tyr tyr tyr tyr ala lys 4591/1531

4521/1541

4551/1551 AGG GGA GGC TAT GGT AAC TAC TAT GCT ATG GAC TAC TGG GGT CAA GGA ACC TCA GTC ACC GTC TCA GGT GGA GGC GGT TCA GGC GGA arg sly gly tyr gly asm tyr tyr ala met asp tyr trp gly gln gly thr ser val thr val ser ser gly gly gly gly ser gly gly 4681/1561 4741/1581 4741/1581 4741/1581 675 GGC GGA TOG GGC ATT GTG ATC ACC CAS TOT CAC ANA TTC ATG TCC ACA TCA GGA GGA GAC AGG GTC AGC ATC ACC GIY GIY ser GIY GIY GIY Ser asp ile val met thr Gln ser his lys phe met ser thr ser val gly asp arg val ser ile thr 4771/1591 4831/1611 THE AND GOT ANT CAS GAT STE AST ACT SET STA SEC THE TAT CAA CAA AAA CCA CCC CAA TET CET AAA CCA ATT THE THE SEC SEA TEE cys lys ala ser gln asp val ser thr ala val ala trp tyr gln gln lys pro gly gln ser pro lys leu leu ile tyr trp ala ser 4861/1621 4891/1631 4921/1641 ACT CES CAC ACT GGA GTC CCT GAT CGC TTC ACA GGC AGT GGA TOT GGG ACA GAT TAT ACT CTC ACC ACT ACC AGT GTG CAG GCT GAA GAC thr arg his thr gly val pro asp and phe thr gly ser gly ser gly thr asp tyr thr leu thr ile ser ser val gln ala glu asp 4951/1651 4981/1661 5011/1671 CTG GCA CTT TAT TAC TGT CAG CAA CAT TAT AGC ACT CCT CCG ACG TTC GGA GGC ACC AAG CTG GAA ACC AAA CGG GCT CCC GGG leu ala leu tyr tyr cys gln gln his tyr ser the pro pro the phe gly gly gly the lys leu glu ile lys are ala pro gly gly I CDR des régions variable et VL fin Serv

pACgp67-ScFv461 -> 1-phase Translation

Fig. 1C

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(504) /2481 STOP 5071/1691 5101/1701 tgt (AA) aga tet gat eeT TTC CTG GGA CCC GGC AAG AAC CAA AAA CTC ACT CTC TTC AAG GAA ATC CGT AAT GTT AAA CCC GAC ACG ATG CYB (CCH) arg ser asp pro phe leu gly pro gly lys asm gln lys leu thr leu phe lys glu ile arg asm vai lys pro asp thr met 3131/1711 5191/1731 SISSIVE STEED AND GEN lys leu val val cly trp lys gly lys clu the try arg glu thr trp thr-arg phe met glu asp ser phe pro ile val asn asp gln 5221/1751 5251/1751 GAA CTG ATG GAT GTT TTC CTT GTT GTC AAC ATG COT CCC ACT AGA CCC AAC CGT TGT TAC AAA TTC CTG GCC CAA CAC GCT CTG CGT TGC Glu val met asp val phe leu val val asn met arg pro thr arg pro asn arg cys tyr lys phe leu ala gln his ala leu arg cys 5341/1781 5371/1791 GAC CCC GAC TAT GTA CCT CAT GAC GTG ATT AGG ATC GTC GAG CCT TCA TGG GTG GGC AGC AAC AAC GAG TAC CCC ATC AGC CTG GCT AAG asp pro asp tyr val pro his asp val ile arg ile val glu pro ser trp val gly ser asm asm glu tyr arg ile ser leu ala lys 5401/1801 5431/1911 5461/1821 aag goc goc toc cca ata atg aac cit cac tot gag tac acc aac tot gaa cag tit atc gat cot got atc tot gag aac tic lys gly gly gly cys pro ile met asm leu his ser glu tyr thr asm ser phe glu glm.phe ile asp arg val ile trp glu asm phe 5521/1841 5551/1851 THE AMS CCC ATC GIT THE ATC GGT ACC GAC TOT GCT GAA GAG GAG GAA ATT CTC CTT GAA GIT TOC CTG GIG TIC AAA GIA AMG GAG TIT tyr lys pro ile val tyr ile gly thr asp ser ala glu glu glu glu ile leu leu glu val ser leu val phe lys val lys glu phe 5581/1981 5611/1871 5641/1851 SCA CCA GAC SCA CCT CTG TTC ACT SCT CCG GCG TAT TAA AAC ACG ATA CAT TGT TAT TAC ATT TAT TAA GCG CTA GAT TCT GTG CGT als pro asp als pro lew phe thr gly pro als tyr OCH asn thr ile his cys tyr AMB tyr ile tyr OCH als lew asp ser val arg 5701/1901 5731/1911 TOT TOA TIT ACA GAC AAT TOT TOT ACG TAT TIT AAT AAT TOA TEA AAT TIA TAA TOT TEA GGG TGG TAT GIT AGA GCG AAA ATC AAA TGA cys OPA phe thr asp asn cys cys thr tyr phe asm asn ser leu asn leu OCH ser leu gly trp tyr val are ala lys ile lys OPA 5761/1921 5791/1931 5821/1941 THE TOA GOG TOT THA THE CHG ANT THE ANT ATT AND TOC TOA ATA GHT THE THA ANT AGE THE COA THE GIT TOA AND AND GOT TOT THE phe ser als ser leu tyr leu asn leu asn ile lys ser ser ile asp leu OCH asn any phe arg leu val ser asn lys gly cys phe 5851/1951 5881/1961 5911/1971 THE GAA CES ATG GET GEA CEA TET AAT GGA TIT TEG CTC AAC GEC ACA AAA CIT GEC AAA TET, TOT AGE AGE AAT CTA GET TIG TEG ATA ser glu pro met ala gly leu ser asm gly phe ser leu asm ala thr lys leu ala lys ser cys ser ser asm leu ala leu ser ile 5941/1981 5971/1991 6001/2001 THE GIT TOT THE TIT TOT AAT AAA GOT TEG ACG TEG THE AAA ATA THA TEE GET TIT GTA TIT CIT TEA TEA CIG TEG TIA GIG TAC phe val cys val leu phe cys esn lys gly ser the ser phe lys ile leu cys als phe val phe leu ser ser leu ser leu val tyr 6031/2011 6061/2021 6091/2031 ANT TEA CTC GAC GTA AAC ACG TTA AAT AAA GCT TGG ACA TAT TTA ACA TGG GGC GTG TTA GCT TTA TTA GGC CGA TTA TGG TGG TGG ash OPA leu asp val ash the leu ash lys ala tep the tyr leu the ser gly val leu ala leu leu gly arg leu ser ser ser ser 6121/2041 6151/2051 6181/2061 CAR CCC TOG TOG TOR GAR GIT GCT TOC GAR GAC GAC TIT GCC AIR GCC ACA CGA CGC CIA TIA ACT GTG TOG GCT AAC ACG TOC GCG ATC gin pro ser ser leu glu val ala ser glu esp asp phe ala ile ala thr arg arg leu leu ile val ser ala asn thr ser ala ile 5211/2071 6271/2091 6241/2081 AAA TIT GIA GIT GAG CIT TIT GGA AIT ATT TOT GAT TGC GGG COT TIT TGG GCG GGT TIC AAT CIA ACT GIG CCC GAT TIT AAT TCA GAC lys phe val val glu leu phe gly ile ile ser asp cys gly arg phe trp ale gly phe asn leu thr val pro eep phe asn eer asp 6301/2101 6331/2111 6361/2121 AND ACC THA GAN AGO GAT GOT GOA GOO GOT GOT AND ATT TOA GAD GOO ANA TOT ANT GOO GOO GOT GOT GOA GOT GAT GAT ANA TOT asm thr leu glu ser asp gly ala gly gly gly asm ile ser asp gly lys ser thr asm gly gly gly gly gly ala asp asp lys ser 6351/2131 6451/2151 ACC ATC GGT GGA GGC GCA GGG GGT GGC GGA GGC GGA GGC GGA GGC GGA GGT GGT GGG GGT GAT GCA GAC GGC GGT TTA GGC TCA AAT GTC 6491/2161 .6511/2171 6541/2181 TOT TTA GGC AAC ACA GTC GGC ACC TCA ACT ATT GTA CTG GTT TCG GGC GCC GTT TTT GGT TTG ACC GGT CTG AGA CGA GTG CGA TTT TTT Ser leu gly asn thr val gly thr ser thr ile val leu val ser gly ala val phe gly leu thr gly leu arg arg val arg phe phe 6571/2191 6501/2201 6631/2211 TEG TTT CTA ATA GET TEE AAC AAT TET TET ETG TEG AAA GET GEA GEG GET TEA GET TEE GTE GEE ATT GET GEA GEG GEE AAT ser phe leu ile ala ser asm asm cys cys leu ser ser lys gly ala ala gly OPA gly ser val gly ile gly gly ala gly gly asm 6691/2231 6721/2241 LCY CYC, YIC CYL COL COL COL COL COL CCY CCY YLE LEY CCC YCE CCY CYY CLL COL CCC CCL CCC CCL YLY YLL LOL 6751/2251 TET GET TTA GIT TET TES COC ACE ATT GTE GOC ACE GOC GCA GGC GCT GGC TGC ACA ACE GAA GGT CGT CTC CTT CGA GGC AGC GCT ser gly leu val cys ser arg thr ile val gly thr gly ala gly ala ala gly cys thr thr glu gly arg leu leu arg gly ser ala 6841/2281 6871/2291 6801/2301 THE GET GET GEC AAT ITA ATA TITA TAA TITE GAA TAC AAA TCG TAA AAA TCT GCT ATA AGC ATT GTA ATT TGG CTA ICG TITT ACC GTG CCG trp gly gly asm ser ile leu CCR leu glu tyr lys ser CCH lys ser ala ile ser ile val ile ser leu ser phe thr val pro 6931/2311 6961/2321 6991/2331 ATA TIT AAC AAC CGC TCA ATG TAA GCA ATT GTA TIG TAA AGA GAT TGT CTC AAG CTC CGC ACG CCG ATA ACA AGC CTT TTC ATT TIT ACT ile phe asn asn ang ser met OCH ala ile val leu OCH ang asp cys leu lys leu ang the pro ile the ser leu phe ile phe the 7021/2341 7051/2351 7081/2361 ACA GCA TTG TAG TGG CGA GAC ACT TGG CTG TCG TCG ACG TAC ATG TAT CCT TTG TCA AAA ACG TCG TTG CCA ACC TTT AAA ATA TTT thr ala leu AMB trp arg asp thr ser leu ser ser thr tyr met tyr ala leu leu ser lys thr ser leu ala ser phe lys ile phe 7111/2371 7171/2381 AAA AGA ACA TOT CTG TTC AGC ACT GTG TTG TCG TAA ATG TTG TTT TTG ATA ATT TGG GCT TCG GCA GTA TCG ACA CGT TCA AAA AAT lys arg thr ser leu phe ser thr thr val leu ser OCH met leu phe leu ile ile cys ala ser ala val ser thr arg ser lys ash 7201/2401 7231/2411 7261/2421 TEX TOC GCX TCX ATT TTG TTG TTG CTX TEX TTG AAT AAX TAX GAT TOT ACA GAT TCX TAT CTX CGA TTG GTC ATG GCC ACC ACA AAT GCT OPA tys als ser ile leu leu phe leu leu leu ast lys OCH asp cys thr asp ser tyr leu arg phe val met als thr thr asm als 7291/2431 7321/2441 7351/2451 ACG CTG CAA ACG CTG GTA CAA TIT TAC GAA AAC TEC AAA AAC GTC AAA ACT CGG TAT AAA ATA ATC AAC GGG CGC TTT GGC AAA ATA TCT thr leu gln thr leu val gln phe tyr glu asn cys lys asn val lys thr arg tyr lys ile ile asn gly arg phe gly lys ile ser 7411/2471 7441/2481 ATT TTA TOG CAC AAG COC ACT AGO AAA TIG TAT TIG CAG AAA ACA ACT TOG GOG CAC AAT TIT AAC GOT GAC GAA ATA AAA GIT CAC CAG ile leu ser his lys pro thr ser lys leu tyr leu gln lys thr ile ser ala his am phe am ala amp glu ile lys val his gln 7471/2491 7501/2501 TTA ATG AGG GAC CAC CCA AAT TIT ATA AAA ATC TAT TIT AAT CAC GGT TCC ATC AAC CAA GTG ATG GAC TAC ATT GAC TGT leu met ser asp his pro asm phe ile lys ile tyr phe asm his gly ser ile asm asm glm val ile val met asp tyr ile asp cys 7561/2521 7591/2531 7621/2541 CCC GAT TTA TIT GAA ACA CTA CAA ATT AAA GGC GAG CTT TCG TAC CAA CTT GTT AGC AAT ATT ATT AGA CAG CTG TGT GAA GCC CTC AAC pro asp leu phe glu thr leu glm ile lys gly glu leu ser tyr glm leu val ser asm jle ile arg glm leu cys glu ala leu asm

3/21

Fig. 1D pACgp67-ScFv461 -> 1-phase Translation 16/01/1 22:01:48 Page 4 7681/2561 7711/2571 GAT TIG CAC AAG CAC AAT TIC ATA CAC AAC GAC ATA AAA CIT GAA AAT GIC TEA TAT TIC GAA GCA CIT GAT CGC GIG TAT GIT TGC GAT asp leu his lys his asm phe ile his asm asp ile lys leu glu asm val leu tyr phe glu ala leu asp arg val tyr val cys asp 7741/2581 7801/2501
TAC GGA TIG TGC AAA CAC GAA AAC TCA CTT AGC GTG CAC GAC GGC ACG TTG GAG TAT TTT AGT CCG GAA AAA ATT CGA CAC ACA ACT ACG 7771/2591 tyr gly leu cys lys his glu asn ser leu ser val his asp gly thr leu glu tyr phe ser pro glu lys ile arg his thr thr met 7831/2611 7891/2531 CAC GIT TOG TIT GAC TGG TAC GCG GCG TGT TAA CAT ACA ACT TGC TAA CCG Gcg gct cGT AAT CAT GGT CAT AGC TGT TRC CTG TGT GAA his val ser phe asp trp tyr ala ala cys CCH his thr ser cys OCH pro ala val arg asn his gly his ser cys phe leu cys glu 7921/2641 7951/2551 7981/2661 ATT GIT ATC CGC TCA CAA TTC CAC ACA ACA TAC GAG CCG GAA GCA TAA AGT GTA AAG CCT GGG GTG CCT AAT GAG TGA GCT AAC TCA CAT ile wal ile arg ser gln phe his thr thr tyr glu pro glu ala CCH ser wal lys pro gly wal pro asn glu OFA ala asn ser his 8011/2671 8041/2551 8071/2691 TAA TIG COT TOC GCT CAC TOC CCG CIT TCC AGT CGG GAA ACC TOT CCT GCC AGC TGC ATT AAT GAA TCG GCC AAC GCG GGA GAG GCC OCH leu arg cys ala his cys pro leu ser ser arg glu thr cys arg ala ser cys ile asn glu ser ala asn ala arg gly glu ala 3101/2701 5131/2711 8161/2721 GIT TOO GIA THE GGC GCT CIT CCG CIT CCT CGC TCA CTG ACT CGC TGC GCT TGC GGC TGC GGC GAG CGG TAT CAG CTC ACT CAA val dys wal led gly ala led pro led pro arg ser led thr arg dys ala arg ser phe gly dys gly glu arg tyr gln led thr glm 8221/2741 8251/2751 AGG CGG TAA TAC GGT TAT CCA CAG AAT CAG GGG ATA ACG CAG GAA AGA ACA TGT GAG CAA AAG GCC AGC AAA AGG CCA GGA ACC GTA AAA arg and OCH tyr gly tyr pro gln asm gln gly ile thr gln glu and thr cys glu gln lys ale ser lys and pro gly thr wal lys 8281/2761 8311/2771 8341/2781 AGG CCG CGT TGC TGG CGT TIT TCC ATA GCC TCC GCC CCC CTG AGG AGC ATC ACA AAA ATC GAC GCT CAA GTC AGA GGT GGC GAA ACC CGA arg pro arg cys trp arg phe ser ile gly ser ala pro leu thr ser ile thr lys ile asp ala gln val arg gly gly glu thr arg 8371/2791 8401/2801 8431/2811 CAG GAC TAT AAA GAT ACC AGG CGT TTC CCC CTG GAA GCT CCC TCG TCC CCC CTG TTC CGA CCC TGC CGC TTA CCG GAT ACC TGT CCG gln asp tyr lys asp thr arg arg phe pro leu glu ale pro ser cys ala leu leu phe arg pro cys arg leu pro asp thr cys pro 8461/2821 8491/2831 8521/2841 CCT TTC TCC CTT CGG GAA GCG TGG CGC TTT CTC ATA GCT CAC GCT GTA GCT ATC TCA GTT CGG TGT AGG TCG TTC GCT CCA AGC TGG GCT pro phe sex leu arg glu ala trp arg phe leu ile ela his ala val gly ile ser val arg cys arg ser phe ala pro ser trp ala 8581/2861 8611/2871 STG TOC ACG AAC CCC CCG TTC AGC CCG ACC GCT GCG CCT TAT CCC STA ACT ATC STC TTG AGT CCA ACC CGG TAA GAC ACG ACT TAT CCC val cys thr ast pro pro phe ser pro thr ala ala pro tyr pro val thr ile val leu ser pro thr ary OCH asp thr thr tyr ary 8641/2881 8671/2891 8701/2901 CAC TOG CAG CAG CCA CTG GEA ACA GGA TEA GCA GAG CGA GGT ATG TAG GCG GTG CTA CAG AGT TCT TGA AGT GGT GGC CTA ACT ACG GCT his trp gln gln pro leu val thr gly leu ala glu are gly met AMB ala val leu gln ser ser OPA ser gly gly leu thr thr ala 5731/2911 8761/2921 3791/2931 ACA CIA GAA GGA CAG TAT TTG GTA TCT GCG CTC TCC TGA AGC CAG TTA CCT TCC GAA AAA GAG TTG GTA GCT CTT GAT CCG GCA AAC AAA thr leu glu gly gln tyr let val ser ala leu cys OPA ser gln leu pro ser glu lys glu leu val ala leu asp pro ala asn lys 8821/2941 8851/2951 8881/2961 CCA CCG CTG GTA GCC GTG GTT THT TTG TTT GCA ACC ACC AGA TTA CGC GCA GAA AAA AAG GAT CTC AAG AAG ATC CTT TGA TCT TTT CTA pro pro leu val ala val val phe leu phe ala ser ser arg leu arg ala glu lys lys asp leu lys lys ile leu OPA ser phe leu 8941/2981 8971/2991 COS COT CTG ACG CTC ACT GGA ACG AAA ACT CAC GTT AAG GGA TIT TOO TCA TGA GAT TAT CAA AAA GGA TCT TCA CCT AGA TCC TIT TAA arg 5ly leu the leu ser gly the lys the his val lys gly phe tep ser OPA asp tyt gln lys gly ser ser pro arg ser phe OCE 9031/3011 9061/3021 ATT AND ANT GAN GIT TIN ANT CAN TOT AND GIN THE NIG NOT AND CIT GOT CIG NOW GIT NOC ANT GOT TAN TON GIG NOG CAC CITA TOT ile lys asm giu val leu asm gim ser lys val tyr met ser lys leu gly leu thr val thr esm ala OCH ser val arg his leu ser 9121/3041 9151/3051 CAG CGA TOT GTC TAT TTC GTT CAT CCA TAG TTG CCT GAC TCC CCC TCG TGT AGA TAA CTA CGA TAC GGG AGG CCT TAC CAT CTG GCC CCA gln and servel the but his pro AMB leu pro asp ser pro ser the and OCH leu and the the als the his leu als pro 9211/3071 9241/3051 OTTO CTG CAA TOA TAC CGC GAG ACC CAC GCT CAC CGC CTC CAG ATT TAT CAG CAA TAA ACC AGC CAG CGG GAA GGG CCG AGC GCA GAA GTG val leu gin OPA tyr arg glu thr his ala his arg leu gin ile tyr gin gin OCH thr ser gin pro glu gly pro ser ala glu val 9301/3101 9331/3111 GTC CTG CAA CTT TAT CCG CCT CCA TCC AGT CTA TTA ATT GTT GCC GGG AAG CTA GAG TAA GTA GTT CGC CAG TTA ATA GTT TGC GCA AGG val leu gin leu tyr pro pro pro ser ser leu leu ile val ala giy lys leu glu CCH val val ary gin leu ile val cys ala thr 9391/3131 TIG TIG COA TIG CIA CAG GCA TOG TGG TGT CAC GCT CGT CGT TIG GIA TGG CIT CAT TOA GCT CGG GIT CCC AAC GAT CAA GGC GAG TIA leu leu pro leu leu gin ala ser trp cys his ala arg arg leu val trp leu his ser ala pro val pro ass asp gin gly glu leu 9421/3141 9481/3161 9511/3171 CAT GAT CCC CCA TOT TOT GCA AAA AAG CGC TTA OCT CCT TCG GTC CTC CGA TCG TTG TCA GAA GTA AGT TGG CCG CAG TGT TAT CAC TCA his asp pro pro cys cys ala lys lys arg leu ala pro ser val leu arg ser leu ser glu val ser trp pro gln cys tyr his ser 9571/3191 9601/3201 THE TIM THE CAS CAS THE ATM ATT CITE TIM CITE TEM THE CAT COS TAM CAS NOT TITT CITE TEM CITE AND ACT CAM COM ACT CAT TOT amp leu trp gln his cys ile ile leu leu ser cys his pro OCH asp ala phe leu OPA leu val ser thr gln pro ser his ser 9661/3221 SAS ANT AST STA TSC GGC GAC CGA GTT GCT CTT GCC CGG CST CAA TAC GGG ATA AFA CGG CGC CAC ATA GCA GAA CTT TAA AAG TGC TGA Glu ean ser val cys gly asp arg val ala leu ala arg arg gln tyr gly ile ile pro arg his ile ala glu leu CGH lys cys ser 9691/3231 9751/3251 9761/3261 TCA THE GAA ARC GIT CIT COG GGC GAA ARC TCT CAR GGA TCT TAC CGC TOT TGA GAT CCA GIT CGA TGT ARC CCA CTC GTG CAC CCA ACT ser leu glu asn val leu arg gly glu asn ser gln gly ser tyr arg cys OPA asp pro val arg cys asn pro leu val his pro the 9841/3261 9871/3291 GAT CIT CAG CAT CIT TEA CIT TEA CCA GCE TIT CIG GGT GAG CAA AAA CAG GAA GGC AAA AIG CCG CAA AAA AGG GAA TAA GGG CGA CAC asp leu gin his leu leu ser pro ala phe leu gly glu gln lys gin glu gly lys met pro gln lys arg glu QCH gly arg his 9901/3301 9931/3311 9961/3321 GGA AND GIT GAN TAC TOA TAC TOT TOO TIT TIC AND ATT ATT GAN GON THE ATC AGG GTT ATT GIC TON TGN GCG GAT ACA TAT TIES AND gly asn val glu tyr ser tyr ser ser phe phe asn ile ile glu ala phe ile ary val ile val ser OPA ala asp thr tyr leu asn 10021/3341 10051/3351 GTA TIT AGA AAA ATA AAC AAA TAG GGG TTC CGC GCA CAT TIC CCC GAA AAG TGC CAC CTG ACG TCT AAG AAA CCA TEA TTA TCA TGA CAT val phe arg lys ile asn lys AMB gly phe arg ala his phe pro glu lys cys his leu thr ser lys lys pro leu leu ser OPA his 10081/3361 10111/3371 10141/3361 THA CET ATA ANA ATA GGC GTA TOA CGA GGC CET TTC GTC TCG CGT GTT TCG GTG ATG ACG GTG ANA ACC TCT GAC ACA TGC AGC TCC CGG

OCH pro ile lys ile gly vai ser ang gly pro phe val ser ang val ser val met thr val lys thr ser asp thr dys ser ser ang

YEY CER LEY CYC CLL CLC LEL YYE COR YLE CCE CCY CTY CYC YYE CCL CLC YER CCC CLL CYC CCC CLC LLC CCC CLL CCC CCL CCC CLL CCC CLL CCC CLL CCC CLL CCC CCL CCC CCL CCC CCL CCC CCL CCC CCL CCC CC

10231/3411

10201/3401

pACgp67-ScFv461 -> 1-phase Translation

Fig IE

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DNA StriderTM 1.2 ### Mardi 16 janvier 2001 21:58:26

pACgp67-ScFv350 -> 1-phase Translation

TA sequence 19511 b.p. AAGCTTTACTCG ... ACCCCCAGTGCC linear

Fig 2

| | | | | | | | | 31/11 | | | | | | | | | 61/ | 21 | | | | | | | | |
|--|--|--|--|--|--|--|---|--|--|--|--|--|--|--|--|---|--|--|--|---|--|--|--|--|--|--|
| AAG CTT TAC | 200 | TAA | AGC | GŸC | TIG | AAG | Cat | CAT AT | CAR C | 7776 | CGT | TTA | TGA | GAT | AAG | ATT | GAA | AGC | ACG | 101 | ` AAA | ATG | . Tri | . 000 | · cc: | CGT |
| lys leu tyr | ser | OCH | ser | glu | leu | lys | ssp | his il | e ame | leu | 2.9 | leu | OPA | szö | jàz | ile | | | thr | cla | ; Āz | met | phe | Pro | ala | arg |
| 91/31 TGG CAC AAC | TAT | TTA | CAA | TEE | GG- | 622 | ململت | 121/41 | | شعمت | 337 | ببب | 3 2 | شعيك | | 323 | 151 | /51 | - | | | - C1- | **** | | | - |
| tro his esn | tyr | leu | gla | cys | gly | gln | val | ile ly | . 250 | 567 | <i>ಆರು</i> | leu | ile | CVS | Dine. | lvs | | cro | leu | 230 | 220 | alu | leu | one: | . ola | 734 |
| 181/61 | | | | | | | | 211/71 | | | | | | | | | 24: | /81 | | | | | | | | |
| GIG ACT AGC | GAA | GAA | GAT | cic | TGG | ACC | GCA | GYY CY | ATA E | GEA | AAA | CAA | AAC | CCT | AGT | ATT | CCA | GCA | ATA | ATC | CAT | LIY | ACC | * *** | : ACG | TET |
| val thr ser | glu | 912 | ಕಿತ್ರಾ | val | crp | thr | ala | glu gl | : ile | AST. | $1\lambda z$ | 2Ju | 823 | bro | ser | ile | | | | ile | asp | leu | thr | 8.57 | chr | ser |
| 271/91 AAA TAT TAT | CAT | GGT | cre | CAT | بلعتمك | تكنيث | cac | 301/10 | | ···· | m=C | 333 | | | CAA | CT3 | | /111 | | ٠, | | ~~ | | GAA | 100 | **** |
| lys tyr tyr | asp | gly | val | his | phe | leu | ಷ್ | ala gl | / leu | leu | EAT. | lvs | lvs | ile | cla | val | EZO. | alv | ola | 76. | leu | DIO | 550 | clu | ser | ile |
| 351/121 | | | | | | | | 391/13: | l. | | • | | | | | | 421 | /141 | | | | | | | | |
| GIT CAA GAA | 212 | TTA | GYC | ACG | GEA | AAA | GYY | TIT AC | GAA | AAG | 101 | CCC | GGC | AIG | TIG | ಯ | ထင | STG | CAC | TGC | ACA | CYC | CCT | ATT | AAT | α |
| val gln glu | phe | ile | ಷ್ಣಾ | 5,5 | val | lys | glu | | | ĮУS | c.À.a | bro | 2,7A | met | leu | val | | | his | cas | thr | his | gly | 11e | asn | 922 |
| ACC GOT TAC | ATG | CTICS | 766 | AGA | TAT | لانبك | 2477 | 481/16 | | ښې | 3 77 | ~ | ~~ | C30 | CAA | | | /171 CXT | 353 | | C3.8 | | | 100 | ~ | C1C |
| thr gly tyr | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 541/181 | | | | | | | | 571/19: | L | | | | | | | | 501. | /201 | | | | | | | | |
| AAA ATT GAA | AGA | CYY | aat | TAC | CII | CAA | CYI | TTA TE | TTA | TAA | LIY | ATA | TIA | 2.22 | œλ | 235 | TIT | AAC | AAA | TAC | Lini | ATC | CTA | 1 | CY. | AAT |
| lys ile glu 631/211 | sra | ستق | 252 | cyr | val | gin | asp | | | Œ | leu | 11e | leu | phe | ala | phe | | | lys | căz | phe | ile | leu | pne | ser | ೭೮೧ |
| TET TEC CCT | TCT | TCC | AGC | GAA | CCA | 444 | ~::A | 551/22: | | 777 | سنجله | **** | 720 | | בידים | خميم | | /231 | ~~ | جيب | | TCC | 227 | ~~ | | TAG |
| cys cys ela | sez | ser | ser | c2u | pro | lys | leu | cvs phe | ala | - T- | ser | val | AM2 | leu | val | ala | aso | cln | tro | are | -27 | ser | asn | 220 | arc | AMS |
| 721/241 | | | | • | | | | 751/25 | Ļ | | | | | | | | 781. | /261 | | | | | | | | |
| GAT TAG GCC | œγ | TAT | يتىت | CCY | CCA | CYY | 1G. | TOC CY | ′ ೧೦೧೭ | TGA | TGT | TAC | c:: | TAT | SCT | LLL | ∞ा | LLL | CCA | ೧೭೭ | ನಿಧ್ಯ | TCT | TTT | GGC | ಞ | AAT |
| asp AMB ala 811/271 | gīĀ | cli | ser | DIO | pro | 2,TU | cys | 241 (25) | arg | 059 | cla | chi | ral | î)î | ala | phe | | | Dro | 9.E | عطت | ser | phe | ala | T.C | ∞ 4 |
| TAG CCG TAA | ACG | TAG | 750 | CGT | csc | GCG | ፈግጉ ል | 641/29 | | | מענם | | 777 | | ملخت | ; ~~ | =/1/ | /291 | عدد | CAA | ~~: | ~~ | ርኔጥ | ~~ | 201 | 337 |
| AMB pro OCH | the | λœ | CAa | arg | ಚಿತ್ರ | ala | ser | arg the | יילט | DIO | 250 | val | CVS | ala | CVS | 220 | متع | civ | ile | Giu Giu | 220 | aru | aso | CCG | Ehr | 257 |
| 901/301 | | | | | | | | 931/31: | | | | | | | | | 961/ | /321 | • | | | | | | | |
| CCA CCA CTT | TCC | CAA | CTA | TAA | CCCC | TGA. | cci | ecc ce. | . crr | TIT | ICI | œλ | TIA | TIT | œ | مين | 121 | 111 | GCA | TCC | TII | cc | CCA | λCC | ಞ | TGT |
| pro pro leu 991/331 | crp | åти | ieu | asr. | erg | CPA | pro | 1021/34 | leu | phe | 367 | دنه | les | phe | ATG | leu | | | | 5 | phe | DEO. | 217 | ser | 1 19 | CAS |
| ACA TOC GOT | TTA | GAT | CAG | TCA | TGA | CGC | GCG | | | عمد | CTT | 700 | مت | CGA | بتكت | GCT | 2027 | (/351 | TTTA | TGG | CAA | CGA | TGC | حثت | حمد | TAA |
| the cys gly | leu | ಷ್ | gln | ser | CPA | وسد | ala | OPA pro | ala | 357 | leu | קינו | 520 | arg | ser | ala | cys | pro | ASO | trp | gln | arg | cys. | val | gln | осн |
| 1081/351 | | | | | | | | 1111/37 | 1 | | | | | | | | 1141 | 1381 | | | | | | | | |
| ACT CIT GIT | | | | | | | | | | | | | | | | | | | | | | | | | | |
| thr lee val | in the | ٠ | ديدو | AGT | 210 | وعه | pne · | 1201/40 | | FID | pro | :50 | ala | 212 | 123 | 163 | | 213 [/4]] | | CPA | met | ser | gin | sex | -13 | A-13 |
| TCA CCA ACT | GIT | 100 | TOT | CCT | CCI | ccc | GII | | | cac | CAT | | 3000 | | ~~ | ~~ | | | | | ~ | ~~~ | | | د | 222 |
| | | | | | | | | | | | | | ~~ ` | | | | | \sim | C 1 4 | ~~ | ~~ | 7 | - x r | | | |
| ser pro thr | val | cλ2 | ser | 220 | 220 | | | | | | | | | | | | 25 | ala | leu | | | | | | | |
| 1261/421 | | | | | | bro | val | val OPA 1291/43 | ser 1 | er.A | కానా | | th: | cys | | cys | 1321 | ala /441 | leu | çlu | giu | leu | leu | leu | leu | lys |
| 1261/421 · CCC ATT CTT | GEA | ATT | CTA | ೧೯೮ | c द | pro AÁS | val ∝× | val OFA 1291/43 ATT TGG | ser 1 ACT | ಕ್ಷಾ ಗದಗ | asp TAA | ಬಿದ್ಗಳ ಆಸುತಿ | thr con | CAA CAA | دعة وحنة | ದಿದ ದಿಸಿತ | 2321 2321 | ala /441 ATT | leu TAG | glu TAA | glu TGA | leu cci | CTG CTG | leu TAT | leu | CCT - |
| 1261/421 | GEA | ATT | CTA | ೧೯೮ | c द | pro AÁS | val ∝× | val OPA 1291/43 ATT TGG ile tr; | ser l ACT tir | ಕ್ಷಾ ಗದಗ | asp TAA | ಬಿದ್ಗಳ ಆಸುತಿ | thr con | CAA CAA | دعة وحنة | ದಿದ ದಿಸಿತ | ಕಾತಿ. ಜಜ 1351 ಕಾತಿ | ala 1/441 ATT ile | TAG AMB | glu TAA | glu TGA | leu cci | CTG CTG | leu TAT | leu CCG | CCT - |
| 1261/421 • CCC ATT CTT • ala-ile leu 1351/451 | GTA val | ATT ile | CTA leu | دست معن | ್ಷ ಆಗ್ರ | pro AÁS lys | val | val OPA 1291/43 ATT TGG ile tr; 1381/46 | ser 1 ACT thr | TCA se: | 277 277 e-25 | ser acy sad | thr con ala | CAA Giu | 252 1CY 723 | ಪ್ತಾ ೧೦೦ ೧۷೩ | 1411 ccc 1321 1321 | ala 1/441 ATT ile 1/471 | TAG AMB | glu TAA OCS | glu TGA GPA | leu cck ala | Leu CIS Leu | leu TAT tyr | leu CCG ala | lys ccr ala . |
| 1261/421 · CCC ATT CTT · ala · ile leu 1351/451 CCA AAT ACA ala asn thr | GTA val | ATT ile GGT | CTA leu CGC | 000 متا وي | CGT erg TTT | pro AÁG lys TCA | val | val 08/ 1291/43 ATT TGC ile tr; 1381/46 CGC TGT arg Cys | Ser 1 ACT thr 1 TAG AMB | ett TCA set AGG | 277 277 2004 280 280 280 280 280 280 280 280 280 280 | ecc ser ser sex | thr ccc | CYS GAA giu CAT | الالا الدلا الدلا | 2007 2000 2000 2000 | 1411 ccc 7351 7351 | ala 1/441 ATT ile 1/471 TCT | TAG AMB | glu TAA OCS SCO CAA | ulu ACA ATA | leu cci ala acc | Len CID ATT | TAT TYF TOT | leu CCG ala ATT | lys GCT - ala . TAT |
| 1261/421 CCC ATT CTT ala ile leu 1351/451 CCA AAT ACA ala asm thr 1441/481 | GTA val GCG ala | GCT GCT GCT | CTA leu CGC arg | 620 600 625 625 620 | CGT erg TIT phe | pro XÁG lys TCA TCA ser | val CCA ala CGA arg | val 0F/ 1291/43 ATT TGC ile tr 1381/46 CGC TG/ arg cys 1471/49 | Ser 1 ACT thr 1 TAG AMB | TCA se: AGG arg | 277 277 277 277 277 278 | era ecc ser ecy ecc ecc ecc ecc | 510 600 979 601 601 | CAT CAT his | bye iii ssr iCy iCy | 27A CCY 523 523 CCC CX2 | 1321 CCC arg 1411 TCC trp 1501 | ala 1/441 ATT ile 1/471 TCT ser 1/501 | TAG AMB GCT ala | glu TAA OCA CAA Gln | ulg ADT AFA ATA ile | leu cck ala acc acc | Leu STT ATT 11e | TAT TYF TGT TGT | leu CCG ala ATT ile | lys GCT - ala . TAT TYT |
| 1261/421 • CCC ATT CTT *ala ile leu 1351/451 • CCA AAT ACA ala asn thr 1441/481 **TOT CTA CAT | GTA val GCG ala GAA | GCT GCT GCT FIE | CTA leu CGC arg | 222 222 223 244 244 | CGT erg TIT phe CIT | pro AAG lys TCA ser TAT | val GCA ala CGA arg CAC | val OF/ 1291/43 ATT TGC ile tr; 1381/46 CGC TG/ arg Cys 1471/49 AAA CTC | ACT ACT TAG TAG AMS | TCA ser AGG arg ATT | 222 223 223 223 224 243 243 | AYC ACC ACC ACC ACC ACC ACC ACC | thr ccc pro | CAT his | 2000 2000 2000 2000 2000 2000 2000 200 | 217. 207 207 518 500 500 500 500 | 1201 1201 1201 1411 500 7131 7131 | ala 1/441 ATT ile 1/471 TCT ser 1/501 | TAG AMB GCT ala | TAA OCTI CAA GIII CGA | TGA GPA ATA ile | leu cci ala acc acc ata acc | Teu CTD Leu ATT LLe CCT | CAS LAL LAL LAL LAL | leu CCG ala ATT ile | lys cor thr cor |
| 1261/421 CCC ATT CTT ala ile leu 1351/451 CCA AAT ACA ala asm thr 1441/481 | GTA val GCG ala GAA | GCT GCT GCT FIE | CTA leu CGC arg | 222 222 223 244 244 | CGT erg TIT phe CIT | pro AAG lys TCA ser TAT | val GCA ala CGA arg CAC | val OF/ 1291/43 ATT TGC ile tr; 1381/46 CGC TG/ arg Cys 1471/49 AAA CTC | ACT ACT TAG TAG AMS I | TCA ser AGG arg ATT | 222 223 223 223 224 243 243 | AYC ACC ACC ACC ACC ACC ACC ACC | thr ccc pro | CAT his | 2000 2000 2000 2000 2000 2000 2000 200 | 217. 207 207 518 500 500 500 500 | 250 1321 1321 1411 160 1501 1501 | Ala //441 ATT ile //471 TCT ser //501 TGG | TAG AMB GCT ala CCA pro | TAA OCTI CAA GIII CGA | TGA GPA ATA ile | leu cci ala acc acc ata acc | Teu CTD Leu ATT LLe CCT | CAS LAL LAL LAL LAL | leu CCG ala ATT ile | lys cor thr cor |
| 1261/421 CCC ATT CTT ala ile leu 1351/451 CCA AAT ACA ala asn thr 1441/481 TOT CCA CAT cys leu his 1531/511 CCT CCA CCA | GTA val GCG ala GAA glu CGT | ATT ile GCT gly CAC his | CTA leu CGC arg CTA val | 22 22 23 24 24 25 25 25 25 25 25 25 25 25 25 25 25 25 | CGT erg TIT The CIT leu | pro AAG lys TCA ser TAT tyr | val GCA ala CGA arg CAC his | val 0FA 1291/43 ATT TOC ile tra 1381/46 CGC TOT arg cys 1471/49 AAA CTC lys let 1561/52 CTT CTC | SET ACT TAG TAG AMB I TAT I TAT | TCA ser AGG arg ATT ile | TAA TEA TEA TEA TEA TEA TEA TEA TEA TEA | ETG SET SET GCC G1y AAC eSI ATT | CAC CAS CAS CAS CAS CAS CAS CAS CAS CAS | CAA CAT CAT his TAC AMB | this series of the series of t | tay tay tay tay tay tay | 1321 1321 1411 760 1591 1591 1591 | Ala /441 ATT ile /471 TCT ser /501 TGG LTD (7531 GAG | TAG AMB GCT ala CCA pro | TAA OCH CAA Gln CGA arg | TCA CPA ATA ile ACC thr | leu cci ala ACG thr GGA gly | Teu ATT ile CCT pro ACG | tor cya cya cya tor tor tor | leu CCG ala ATT ile CCT gly GTC | lys GCT - ala - TAT TYT CDC and GAT |
| 1261/421 CCC ATT CTT ala ile leu 1351/451 CCA AAT ACA ala esn thr 1441/481 TOT CTA CAT cys leu his 1531/511 CCT CTA CCA ala leu ala | GTA val GCG ala GAA glu CGT | ATT ile GCT gly CAC his | CTA leu CGC arg CTA val | 22 22 23 24 24 25 25 25 25 25 25 25 25 25 25 25 25 25 | CGT erg TIT The CIT leu | pro AAG lys TCA ser TAT tyr | val GCA ala CGA arg CAC his | val 0F/ 1291/43 ATT TOX ile tr; 1381/46 CGC TOY arg cys 1471/49 AAA CTC 175 leu 1561/52 CTT CTC leu leu | Ser ACT TAG TAG TAT TAT TAT TAT TAT | TCA ser AGG arg ATT ile | TAA TEA TEA TEA TEA TEA TEA TEA TEA TEA | ETG SET SET GCC G1y AAC eSI ATT | CAC CAS CAS CAS CAS CAS CAS CAS CAS CAS | CAA CAT CAT his TAC AMB | this series of the series of t | OCH TAY COL COY COY COC COC CAS | 1321 CSC 429 1411 TSC 1501 CT 1501 1501 1501 1501 1501 1501 1501 150 | Ala /441 ATT ile /471 TCT ser /501 TCG trp /531 GAG glu | TAG AMB GCT ala CCA pro | TAA OCH CAA Gln CGA arg | TCA CPA ATA ile ACC thr | leu cci ala ACG thr GGA gly | Teu ATT ile CCT pro ACG | tor cya cya cya tor tor tor | leu CCG Ala ATT ile CCT Gly GTC | lys GCT - ala - TAT TYT CDC and GAT |
| 1261/421 CCC ATT CTT ala ile leu 1151/451 CCA AAT ACA ala asn thr 1441/481 TOT CCA CAT cys leu his 1531/511 CCT CTA CCA ala leu ala 1521/541 | GTA val GCG ala GAA glu CGT arg | ATT ile GCT gly CAC his ACC thr | CTA leu CGC arg CTA val CCA ala | 617 617 618 618 618 618 618 618 618 618 618 618 | CGT TIT The CIT Leu TGA OFA | DIO AAG IYS TCA SET TAT TYT ACG thr | val GCA ala CGA arg CAC his TAT tyr | Val 0FA 1291/43 ATT TOK ile tri 1381/46 CGC TOM arg cys 1471/49 AAA CTC 1561/52 CTT CTC leu leu 1651/55 | SET IN TAGE IN | TCA ser AGG ATT ATT ile ATT | TAA CCI TAG AMB TIA Leu TAA CCI | TCA Ser Ser Sty AAC ean ATT ile | thr con ala coc pro rer cys crc leu | CAT his TAC AVB CAA gln | TCA ser phe CGA TTT phe | CCH CCH CCH CCH CCH CCH CCH CCH CCH CCH | 1321 CCC 429 1411 TCC 1501 CTC 1501 CTC 1501 CTC 1501 CTC 1501 | Ala /441 ATT ile /471 TCT ser /501 TCG trp /531 CAG glu /561 | TAG AMB GCT ala CCA pro | TAA OCA Gin CGA arg TTT | TCA CPA ATA ile ACC thr TCA CPA | leu cck ala ACG shr GGA gly TAC tyr | Teu CTG Leu ATT LLe CCT pro ACG thr | cha cha cha cha cha cha cha cha cha | leu CCG ala AIT ile CCT gly GTC val | lys GCT - ala - TAT TYT CCC arg GAT asp |
| 1261/421 COC ATT CTT ala ile leu 1351/451 COC AAT ACA ala asn thr 1441/481 TOT CTA CAT Cys leu his 1531/511 CCT CTA CCA ala leu ala 1521/541 TTT CCA ACA | GTA val GCG ala GAA glu CGT arg | ATT ile GCT gly CAC his ACC thr | CTA leu CGC arg CTA val GCA ala | 111 125 125 125 127 127 127 127 127 127 127 127 127 127 | CGT erg TIT phe CIT leu TGA OFA | AAG lys TCA ser TAT tyr ACG thr | val GCA ala CGA arg CAC his TAT tyr AAA | val OP/ 1291/43 ATT TCC ille trj 1381/46 CGC TGT arg Cys 1471/45 AAAA CTTC lys let 1561/55 CTT CTC 161 let 1651/55 CTA AAC | ACT | TCA ser AGG arg ATT ile ATT ile TTG | 244 2001 1144 1144 1144 1144 1144 1144 1 | TOA SOF CONTY AAC ATT ile | thr con ala con pro refr cys cruc leu | CAT his TAG AMB | TCA Ser Tri phe CGA arg | Cys CCC arg GGA gly CCT arg TAA CCE | 1321 CSS 419 1411 TSC 1501 CT pro 1501 CT pro 1501 TST 1501 TST | Ala /441 ATT ile /471 TCT ser /501 TCC tip /531 GAG glu /561 GCG | TAG AMB GCT ala CCA pro CCA pro | TAA OCA CAA Gin CGA arg Tir phe | TCA CPA ATA ile ACC thr TCA CPA TOC | leu cck ala ACG shr GGA gly TAC tyr | Leu CTD Leu AFF LLe CCT pro ACG thr | the star star star star star star star star | leu CCG Ala AIT ile CCT gly GTC val ACA | lys GCT: ala TAT TYT CCC GAT GAT GAT CTC |
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| 1261/421 CCC ATT CTT ala ile leu 1151/451 CCA AACT ACA ala asm the 1441/461 TOT CCA CAT cys leu his 1531/511 CCT CTA GCA ala leu ala 1521/541 CTC CTA GCA phe ala the 1711/571 CTC GTT ATG val val met 1801/601 TAC GGT GCA leu ala ala 1581/651 CGA CGC ACA arg arg the 2071/691 AGT TGG GTT ATG ATG TTT ATG ATG ATG ATG ATG ATG ATG ATG ATG | GTA Val GGG Ala GGA ACT ACT ACT ACT ASS GAT ACT TAA GTT Val TAA TTAA | ATT TICC CYS AACT TILE | CTA lev CGC arg GTA val GCA ala GTT val CAT his CCA ala GTT cys GCC ala GTT val CCC pro | TOP COPY TAGE STAY THE STAY AND TAGE SAY | CTT phe CTT leu MA OPA COS ALA ASC ASSE COS PRO CTA ASC PRO | AND ACT OF ACT O | val GCA ala CGA ala CAC his TAT tyr AAA lys CCC arg GAT GAT GAT GAT GAT GAT GAT GAT GAT GA | Val OR 1291/4; AAAT TOCK 11e Un; 1381/46 CT 1381/46 CT 1471/45 AAA CTC 1471/45 AAA CTC 1561/5; CTA AAC 151/5; CTA AAC 16U ASS 1741/5; GCC GCC GCC GCC GCC GCC GCC GCC AT G | ACT | TOA Ser ACC ACC ACC ACC TOC TOC TOC TOC TOC TOC TOC TOC TOC T | 지 않는 사람 다리 보신 이 없는 것이 다리 영화 연화 전화 문화 전화 | TANGE OF LANGE AND | The Sala Coro Fox Chee Sala Grad AAB Sala AAB Sa | CYS GAA GEIG CATS TAMB CAA ATA TTCU TAAC COTO COTO ATA CAA CAA CAA | TOTAL TITLE CONTROL ATTAILS CO | Cys CCC CCC CCC CCC CCC CCC CCC CCC CCC | 4.75 1121 Cardinal Control Con | Ala (7441) (1471) Tor (7501) (| TAGE TAGE TAGE TAGE TAGE TAGE TAGE TAGE | TAA CAIN CAIR TO CAIN TO CAIN ACT TO CAIN | TOA ATA ATTE TOA ATTE | leu CCA ala ACG ATH GGLY TAXT GCC ala CCC PPTO GCG ala TTC PPTO GCG ALA TT | leu CIB Leu ATT LILe CIT | TAT TOTAL TO | leu CCC Alla AllT ile CCT Gly GTC ACA Alla CCA ACA Alla CCA ATA CCA ATA CCC CCC ATA CCC CCC ATA CCC CC | TATE OF A TOTAL TO |
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Fig. 2B 16/01/1 21:58:26 Page 2 pACgp67-ScFv350 -> 1-phase Translation 2431/811 2491/831 2461/821 ANT TAX ATA OUT TOU GAD GOA ADG TOU AND ATO TOT GOA COW GIT COG GOA GOT TOU ANT GIA ANA ANT TIT TAX GAA GOD AND AND AND OUT ILE also dye asp als the dye the ile dye also are also and also are also led ile ser pie by glu also met the . 2551/851 2591/661 TEA COC COG TAG TEA CAA CEA TOA CEC COA AAA GAA CTO COG ACT ACA AAA TTA COC AGT ATO TOG GTG ACG TTA AAA CTA TTA ASC CAT OPA pro pro AMB OPA gln arg ser arg pro lys glu leu pro thr thr lys leu pro ser met ser val thr leu lys leu leu ser his 2611/871 2641/891 CCA ATC GAC COT TAG TOG AAT CAG GAC CGC TGG TGC GAG AAG CCG CGA AGT ACG GCG AAT GCA TCG TAT AAC GTG TGG AGT CCG CTC ATT pro ile asp arg AMB ser asm glm asp arg trp cys glu lys pro arg ser met ala asm ala ser tyr asm val trp ser pro leu ile 2701/901 2751/921 AGA GCG TCA TGT TTA GAC AAG AAA GCT ACA TAT TEA ATT GAT GCC GAT GAT TIT ATT GAT AAA TTG ACC CTA ACT CCA TAC ACG GTA TTC arg ala ser cys leu amp lym lym ala thr tyr leu ile amp pro amp phe ile amp lym leu thr leu thr pro tyr thr vai phe 2791/931 2851/951 THE ART EGG EGG GIT TIG GIT AAA ATT ICC EGG CIG CGA TIG THE ATG CIG TIA ACG GCT CCC CCC ACT ATT AAT GAA ATT AAA AAT TCC tyr asn gly gly val leu val lys ile ser gly leu arg leu tyr met leu leu thr ala pro pro thr ile asn glu ile lys asn ser 2881/961 2941/981 ANT TIT AAA AAA COC ACC AGC AGG AGA AAC ATT TOT ATG AAA GAA TOC GTA GAA GGA AAG AAA, AAT GTC GTC GAC ATG CTG AAC AAC AAG ACT asn phe lys lys arg ser lys arg asn ile cys met lys glu cys val glu gly lys lys asn val val asp met leu asn asn lys ile 3001/1001 3031/1011 ANT AND COT COG TOT ATA AAA AAA ATA TTG AAC GAT TTG AAA GAA AAC AAT GTA CCG CGC GGC GGT ATG TAC AGG AAG AGG TTT ATA CTA asn met pro pro cys ile lys lys ile leu esn esp leu lys glu esn esn wal pro erg gly gly met tyr erg lys arg phe ile leu 3091/1031 3121/1041 3061/1021 AAC TOT TAC ATT GCA AAC GTG GTT TOG TOT GCC AAG TOT GAA AAC CGA TOT TTA ATC AAG GCT CTG ACG CAT TTC TAC AAC CAC GAC TCC ash dys tyr ile ala ash val val ser dys ala lys dys glu ash arg dys leu ile lys ala leu thr his phe tyr ash his asp ser 3181/1061 3211/1071 AND TOT GTG GOT GAA GTC ATG CAT CTT TTA ATC AAA TCC CAA GAT GTG TAT AAA CCA CCA AAC TGC CAA AAA ACT GTC GAC AAC lys cys val gly glu val met his leu leu ile lys ser glo asp val tyr lys pro pro asn cys glo lys net lys thr val asp lys 3271/1091 3301/1101 CTC TOT CCG TIT GCT GCC AAC TGC AAG GGT CTC AAT CCT ATT TGT AAT TAT TGA AIA AIA AAA CAA TTA TAA AIG CIA AAT TTG TIT TIT leu cys pro phe ala gly asn cys lys gly leu asn pro ile cys asn tyr OPA ile ile lys gln leu CCH met leu asn leu phe phe 3331/1112 3391/1131 ATT AAC GAT ACA AAC CAA ACG CAA CAA CAA CAA CAT TTG TAG TAT TAT CTA TAA TTG AAA ACG CGT AGT TAT AAT CCC TGA GGT AAT ATT CAA ile asm asp thr asm glm thr glm glm glm his lew AMS tyr tyr lew OCK lew lys thr arg ser tyr asm arg OPA gly asm ile OCK 3451/1151 3481/1161 ANT CAT TIT CAA AND AIT CAC AGT TAA TIT GGG ACA ANA THA TIT TAT TIT CAC ANA AAC TAG ACG CCT TGT CGT CTT CTT CCT ACT asn his phe gln met ile his ser OCH phe ala the ile CCH phe tyr phe his ile asn AMB the pro cya and leu leu and ile 3541/1181 3571/1191 3511/1171 CCT TOT CTT TIT CAT TIT TCT CCT CAT AAA AAT TAA CAT AGT TAT TAT CGT ATC CAT ATA TGT ATC TAT CGT ATA GAG TAA ATT TIT TGT pro ser let the his the ser pro his lys asn CCH his ser tyr tyr arg the his the tys the tyr arg the glu CCH lie the cys 3661/1221 3501/1201 3631/1211 TOT CAT ANA THAT ATA TOT CIT TIT THA TOO GOT STA TAG DAC COC TOC GCA TAG TIT TIC TOT ANT THA CAA CAG TOC TAT TIT CIT GTA cys his lys tyr ile cys lau phe CCH trp gly val AMS tyr are tys ala AMS phe phe cys asn lau gin gin cys tyr phe lau vai 3721/1241 3751/1251 GTT CTT CGG AGT GTG TTG CTT TAA TTA TTA AAT TTA TAY AAT CAA TGA ATT TGG GAT CGT CGG TTT TGT ACA ATA TGT TGC CGG CAT AGT val leu arg ser val leu leu OCH leu leu asn leu tyr asn gln CPA ile trp asp arg arg phe tys thr ile tys cys arg his ser 3721/1261 3841/1291 3721/1261 ACC CAG CIT CIT CITA GIT CAA THA CAC CAT TIT TITA GCA GCA CCC GAT, THA CAT AAC TIT GCA AAA TOT TOT ACC AAC COT TAA ACA AAA thr gln leu leu leu val gln leu his his phe leu ala als pro asp OCH his asn phe pro lys cys cys thr asn arg OCH thr lys 3871/1201 3931/1311 . ACA GTT CAC CTC CCT TITT CTA TAC TAT TOT CTG CGA GCA GTT GTT TGT TGT TAX AAA TAA CAG CCA TTG TAA TGA CAC CCA CAC ACT AAT ; thr val his leu pro phe lau tyr tyr cys leu arg ala val val cys cys OCH lys OCH gln pro leu OCH OFA asp-ala gln thr asm... 3961/1321 3991/1331 ATC ACA AAC TOO AAA TOT CTA TCA ATA TAT AOT TOC TGA TAT CAT CGA GAT AAT TAA AAT GAT AAC CAT CTC GCA AAT AAA TAA GTA TIT ile thr asn trp lys cys leu ser ile tyr ser cys CPA tyr his gly asp asn OCH asn asp asn his leu ala asn lys OCH val pho 4051/1351

4081/1361

TAC TOT TIT COT AAC ACT TIT GEA ATA AAA AAA CUT ATA AAT ATE COG GAT TAT TCA TAC COT CCC ACC ACC ACC GGA TET ATG CTA. tyr tys phe arg asn ser the val ile lys lys pro ile asn ile pro asp tyr ser tyr arg pro thr ile gly arg gly ser (med) len 4201/1401 4171/1391 CTA GTA AAT CAG TCA CAC CAA GGC TTC AAT AAG CAA CAC ACA ACC AAG ATG GTA AGG GCT ATT GTC TTA CAT GTG CTT TTG GCC GCG GCG GCG leu val asm gln ser his gln gly phe asm lys glu his thr ser lys net val ser ala ile val leu tyr val leu ala ala ala 4231/1411

GCG CAT TCT GCC TTT GCG/GCG GAT CTT gga tcc CAT CAT CAC CAC CAC CAC CAC ACT gga aga/GAA TTC/CAG GTC CAA CTG CAG CAG TCT ala his ser ala phe alavala asp leu gly ser his his his his his his ile glu gly arg/glu phe gln val gln leu gln gln ser A331/1441 4351/1451 4381/1461 GGG GCT GAA CTG GCA AAA CCT GGG GCC TCA GTG AAG CTG TCC TGC AAG GCT TCT GCC CAC ACC TTT ACT AGC TAC TGC ATG CAC TGG GTA
Gly ala glu leu ala lys pro gly ala ser val lys leu ser cys lys ala ser gly his thr phe thr ser tyr trp met his trp val 4441/1481 4471/1491 AAA CAG AGG CCT GGA CAG GOT CTG GAA TGG ATT GGA TAC ATT AAT CTT AGG AGT GGT TAT ATT AAG TAC AAT CAG GAC ATC AAG lys glm arg pro gly glm gly leu glu trp ile gly tyr ile asm leu ser ser gly tyr ile lys tyr asm glm glu phe lys asp lys 4561/1521 4501/1501 4531/1511 4501/1501

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4501/1501 4591/1531 4621/1541 4651/1551 4711/1571 ->:VL TOT GSC GST GGC GGA TCG GAC ATT GTG ATG ATC CAG TCT CAC AAA TTC ATG TCC ACA TCA GTA GGA GAC AGG GTC AGC ATC ACC TGC AAG Ser gly gly gly ser esp ile val met ile gln ser mis lys phe met ser thr ser val gly esp erg val ser ile thr cye lys 4801/1601 4831/1611 GCC ACT CAG GAT GTG ACT ACT GCT GTA GCC TGG TAT CAA CAA AAA CCA GGG CAA TCT CCT AAA CTA CTG ACT TAC TGG GCA TCC ACC GGG ACT TCT GTA AAA CTA CTG ACT TAC TGG GCA TCC ACC GGG ACT TCT GTA AAA CTA CTG ACT TAC TGG GCA TCC ACC GGG ACT TCT GTA AAA CTA CTG ACT TAC TGG GCA TCC ACC GGG ACT TCT GTA AAA CTA CTG ACT TAC TGG GCA TCC ACC GGG ACT TCT TAC TGG GCA TCC ACC TGG TAC TGG TAC TGG ACT TCC ACC TGG TAC TGG ACT TCC ACC TGG TAC TG 4891/1631 4861/1621 4921/1641 CAC ACT GGA GTC CCT GAT CGC TTC ACA GGC AGT GGA TCT GGG ACA GAT TAT ACT CTC ACC ACT AGC AGT GTG GAG GCT GAA GAC CTG GCA his the gly val pro asp arm phe the gly ser gly ser gly the asp tyr the leu tie ile ser ser val gin ala glu asp leu ala 5011/1671 4951/1651

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Fig. 2C

pA Cgp67-ScFv350 -> 1-phase Translation 16/01/1 21:58:26 Page 5101/1701 5071/1691 AGA LET GRE GET THE CHG GGA COE GGC ANG AND CAN ANN CHE NET CHE THE ANG GAN AND COT ANT GIT ANN COE GAE ANG ANG CHE and ser asp pro phe leu gly pro gly lys asm glm lys leu thr leu phe lys glu ile and asm val lys pro asp thr met lys leu 5131/1711 5191/1731 CTC CTT COA TOO ARA COA ARA GRO THE TRE AGG GRA ACT TOO ACE COC THE ATG GRA-GRE AGE THE COE ATT GIT ARE GRA GRA GRA GIT val val gly trp lys gly lys glu phe tyr arg glu the trp the arg phe met glu asp ser phe pro ile val asm asp glm glu val 5281/1761 5251/1751 5221/1741 ATG GAT GIT TIC CIT GIT GIC AAC AIG COT CCC ACT AGA CCC AAC COT TOT TAC AAA TIC CIG GCC CAA CAC GCT CTG GOT TGC GAC CCC met asp val phe leu val val ash met arg pro thr arg pro ash arg cys tyr lys phe leu ala gln his ala leu arg cys asp pro 5371/1791 5341/1791 5311/1771 GAC TAT GTA COT CAT GAC GTG ATT AGG ATC GTC GAG COT TCA TGG GTG GGC AGC AAC GAG TAC CGC ATC AGC CTG GCT AAG AAG GGC asp tyr val pro his asp val ile arg ile val glu pro ser trp val gly ser asm asm glu tyr arg ile ser leu ala lys lys gly 5431/1811 5461/1621 5401/1601 GGC GGC TGC CCA ATA ATG AAC CTT CAC TCT GAG TAC ACC AAC TCG TTC GAA CAG TTC ATC GAT GGT GTC ATC TGG GAG AAC TTC TAC AAC gly gly cys pro ile met asn leu his ser glu tyr thr asn ser phe glu gln phe ile asp arg val ile trp glu asn phe tyr lys 5491/1831 5521/1841 5551/1851 CCC ATC STT TAC ATC GST ACC GAC TCT GCT GAA GAG GAG GAA ATT CTC CTT GAA GTT TCC CTG GTG TTC AAA GTA AAG GAG TTT GCA CCA pro ile val tyr ile gly thr asp ser ala glu glu glu glu ile leu leu glu val ser leu val phe lys val lys glu phe ela pro 5611/1871 5641/1881 5581/1561 GAC GCA CCT CTG TTC ACT GGT CCG GCG TAT TAA AAC ACG ATA CAT TGT TAT TAG TAC ATT TAT TAA GGG CTA GAT TCT GTG CGT TGT TGA asp ala pro leu phe thr gly pro ala tyr CCH asn thr ile his cys tyr AMB tyr ile tyr CCH ala leu asp ser val arg cys OFA 5571/1991 5731/1911 TIT ACA GAC AAT TOT TOT ACG TAT TIT AAT AAT TCA TTA AAT TTA TAA TCT TTA GGG TGG TAT GIT AGA GCG AAA ATC AAA TGA TIT TCA The thr asp asn cys cys thr tyr phe asn asn ser leu asn leu OCH ser leu gly tro tyr val arg ala lys ile lys OFA phe ser 5821/1941 5791/1931 5761/1921 COG TOT TTA TAT CTG AAT TTA AAT ATT AAA TOC TOA ATA GAT TTG TAA AAT AGG TTT CGA TTA GTT TCA AAC AAG GGT TGT TTT TCC GAA ala ser leu tyr leu asn leu asn ile lys ser ser ile asp leu OCH asn arg phe arg leu val ser asn lys gly cys phe ser glu 5911/1971 5881/1961 5851/1951 CCG ATG GCT GCA CIA TCT AAT GCA TIT TEG CTC AAC GCC ACA AAA CTT GCC AAA TCT TGT AGC AGC AAT CTA GCT TTG TEG AEA TTC GC pro met ala gly leu ser asm gly phe ser leu asm ala thm lys leu ala lys ser cys ser ser asm leu ala leu ser ile phe val 5941/1981 6001/2001 5971/1991 TOT OTT TOT TOT ANY ANA GOT TOG ACG TOG TIT ANA ATA TIA TOC GOT TIT OTA TIT CIT TIA TOA CIG TOG TIA CAAT TOA CYS WAL leu phe cys esn lys gly ser thr ser phe lys ile leu cys ala phe wal phe leu ser ser leu wal tyr asn OFA 6061/2021 6091/2031 5031/2011 CTC GAC GTA AAC ACG TTA AAT AAA GCT TGG ACA TAT TTA ACA TCT GGC GTG TTA GCT TTA TTA GGC CGA TTA TCG TGG TGG CAA CCC leu asp wal asm thr leu asm lya alo trp thr tyr leu thr ser gly wal leu alo leu elu gly arg leu ser ser ser gla pro 6161/2061 6121/2041 6151/2051 TEG TEG TEA GAA GET GET TEE GAA GAE GAE TIT GEE ATA GEE ACA EGA EGE ETA TEA ATT GTG TEG GET ARE ACG TEE GEE AND AND TET ser ser leu glu val ala ser glu asp asp phe ala ile ala thr arg arg leu leu ile val ser ala ash thr ser ala ile lys phe 6271/2091 6241/2081 GIA GIT GAG CIT TIT GGA AIT ATT TOT GAT TGC GGG CGT TIT TGG GCG GGT TTC AAT CTA ACT GTG CCC GAT TIT AAT TCA GAC AAC ACG val val glu leu phe gly ile ile ser asp cys gly are phe trp als gly phe asm leu the val pro asp phe asm ser asp asm the 6331/2111 6361/2121 6301/2101 THA CHA ACC GAT GOT GOA GOC GOT GOT AND ATT TOA CAC GOC ANA TOT ACT ANT GOC GOC GOT GOT GOA GOT GAT GAT ANA TOT ACC AND Jeu glu ser asp gly ale gly gly gly gly ale sep asp lys ser the lie 6421/2141 6391/2131 6541/2181 · 6511/2171 CON AND AND GOT CON AND THE ACT ANT GOT CON GOT GOT GOT GOT GOT GOT THE GOT THE ACE GOT CON AND COA GOT CON THE TOT THE TOT THE GOT WAS GOT CON AND COA GOT CON AND THE TOT THE TOT THE GOT THE ACE GOT CON AND COA GOT CON AND THE TOT THE TOT THE GOT THE ACE GOT CON AND COA GOT CON AND THE TOT THE TOT THE GOT THE ACE GOT CON AND COA GOT CON AND THE TOT THE TOT THE GOT THE ACE GOT CON AND COA GOT CO 6601/2201 6631/2211 5571/2191 CIA AIR CCT TOC AND ANT TOT TOT CIG TOT AND GGT GGA GCG GGT TIGA GGT TICC GTC GGC ANT GGT GGA GGG GGC ANT TOA GAC Leu ile ala ser asn eys cys leu ser ser lys gly ala ala gly OFA gly ser val gly ile gly gly ala gly gly asn ser asp 6661/2221 6691/2231 ATT GAT GOT GOT GOT GOT GOT GOA GOC GOC GOA ATT TIA GOC ACG GOA GAA GOT GOT GOC GOC GOT GOC GOT ATA ACT TOT TOT GOT ile asp gly gly gly gly gly gly gly gly ala gly met leu gly the gly glu gly gly gly gly gly ala ala gly ile ile cym ser gly
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Fig 2.D

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pACgp67-ScFv350 -> 1-phase Translation

Fig 2 L

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10251/3421

10291/3431

10321/3441

ANG CGC CAT CAG AGA AGA THE TAC TGA GAG TGC ACC ATA TGC GGT GTG AAA THE CGC ACA GAT GGG TAA GGA GAA AAT ACC GGA TGA GGC TAT AGG AT GGG TAA GGA GAA AAT ACC GGA TGA GGC TAT AGG AT GGG TAA GGA GAA AAT ACC GGA TGA GGC TAT AGG AT GGG TGC ATT GGC ATT GGC ATA GGC ATT GGC ATA GGC AGG TGC GGC CGT CTT CGC TAT TGA GGC AGG TGG AAAG GGG GAT GTG AAAG GGG GAT GTG TGG AAAG GGC GAT GTG TGG AAAG GGG GAT GTG CGC AGG TGG TGG AAAG GGG GAT GTG CGC AGG GGT TTT CCC AGT CAC GAC GTT GTA AAA GGA GGG CCA GTG CC GGG GTG GTA AAAG GGG GAT GTG GCC AGG GTG GTG AAAG GGG GAT GTG AAAG GGG GAT GTG GGG AAAG GGG GAT GTG AAAG GGG GCC AGTG GCC AGG GTG GTG GTG GTG AAAG GGG GCC AGTG GCC AGG GTG GTG GCC AGG GTG GTG GAT AAAC GGA GGG GCC AGTG GCC AGG GTG GCC AGG GTG GTG GCC AGG GTG GTG GCC AGG GCC AGG GTG GCC AGG GCC AGG GTG GCC AGG GCC

10/21

Figure 3: clonetherap.99B3

VH sequence:

GAGGTGAAGCTTCTCCAGTCTGGAGGTGGCCTGGTGCAGCCTGGAGGATCCCTGA AACTCTCCTGTGCAGCCTCAGGAATCGATTTTAGTAGATACTGGATGAGTTGGGT TCGGCGGGCTCCAGGGAAAGGACTAGAATGGATTGGAGAAATTAATCCAGATAG CAGTACAATAAACTATGCACCATCTCTAAAGGATAAATTCATCATCTCCAGAGAC AACGCCAAAAATACGCTGTACCTGCAAATGAGCAAAGTGAGATCTGAGGACACA GCCCTTTATTACTGTGCAAGAGGACTGGGACAGAACTTTGACTACTGGGGCCAAG GCACCACTCTCACAGTCTCCTCA

VL sequence:

Figure 4: clonetherap.88E10

VH sequence:

GAGGTGAAGCTGGAGTCTGGAGGAGGCTTGGTACAGCCTGGGGGTTCTCTG AGTCTCTCTGTGCAGCTTCTGGATTCACCTTCACTGATTACTCCATGAACTGGGT CCGCCAGCCTCCAGGGAAGACACTTGAGTGGTTGGCTTTTATTAGAAACAAAGCT AATGGTTACACAGCAGAGTACAGTGCATCTGTGAAGGGTCGGTTCTCCATCTCCA GAGATAATTCCCAAAGCATCCTCTATCTTCAAATGAATGCCCTGAGAGCTGAGGA CAGTGCCACTTATTACTGTGCAAGGGGATGGTATGCTATGGACTACTGGGGTCAA GGAACCTCAGTCACCGTCTCCTCA

VL sequence:

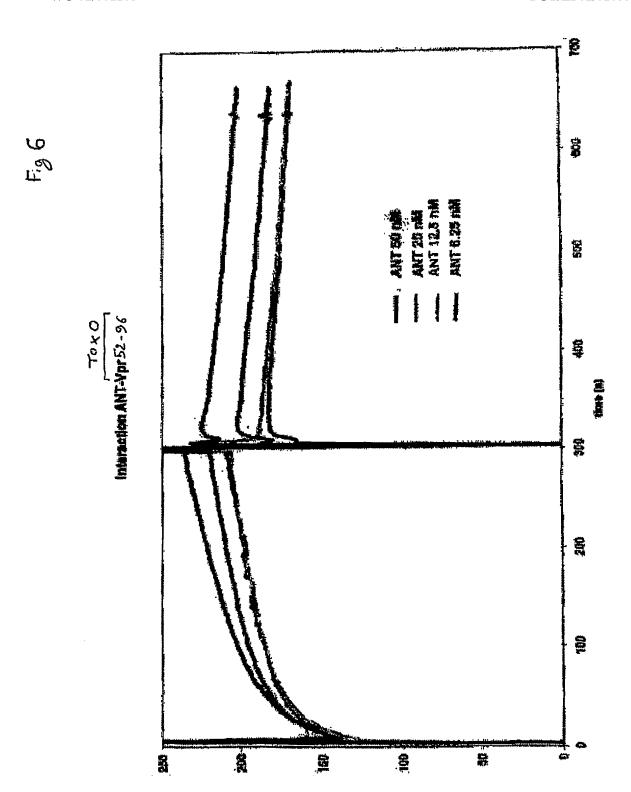
Figure 5: clonetherap.152C3

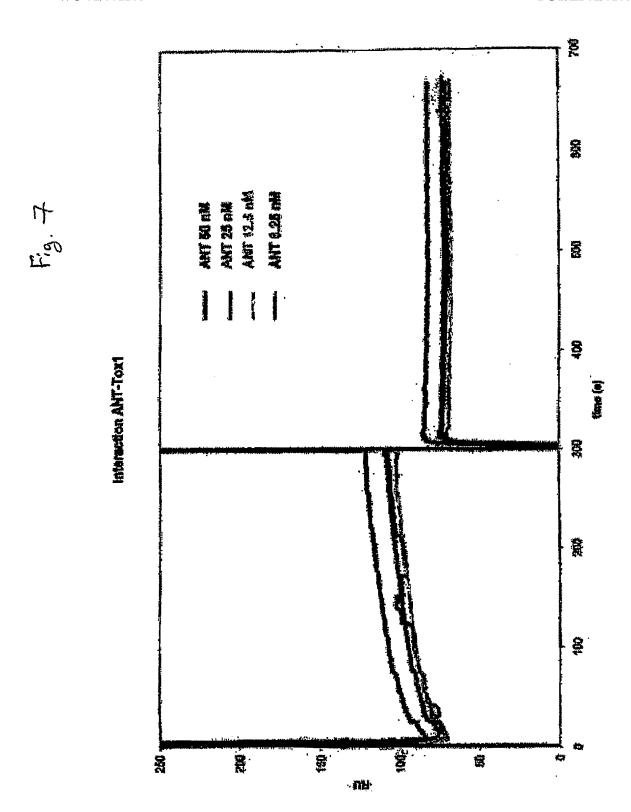
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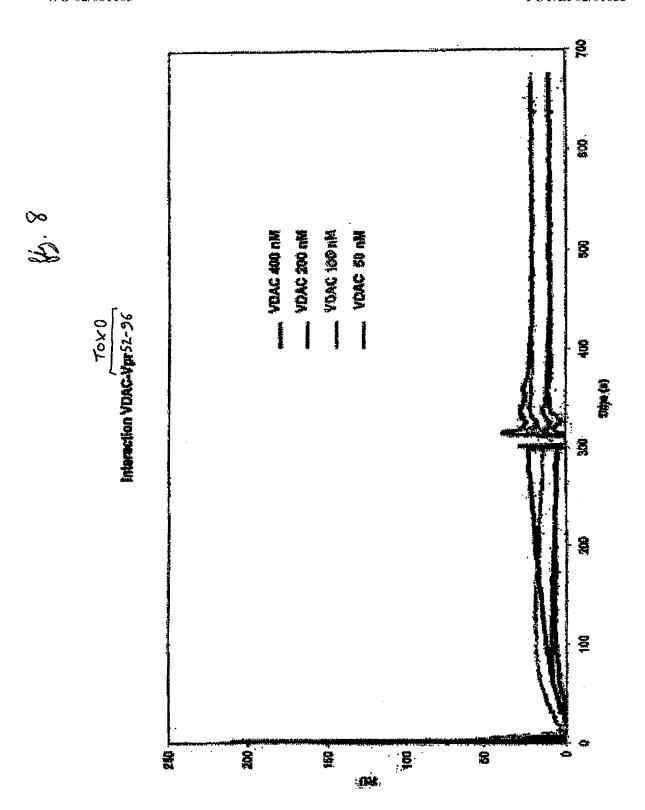
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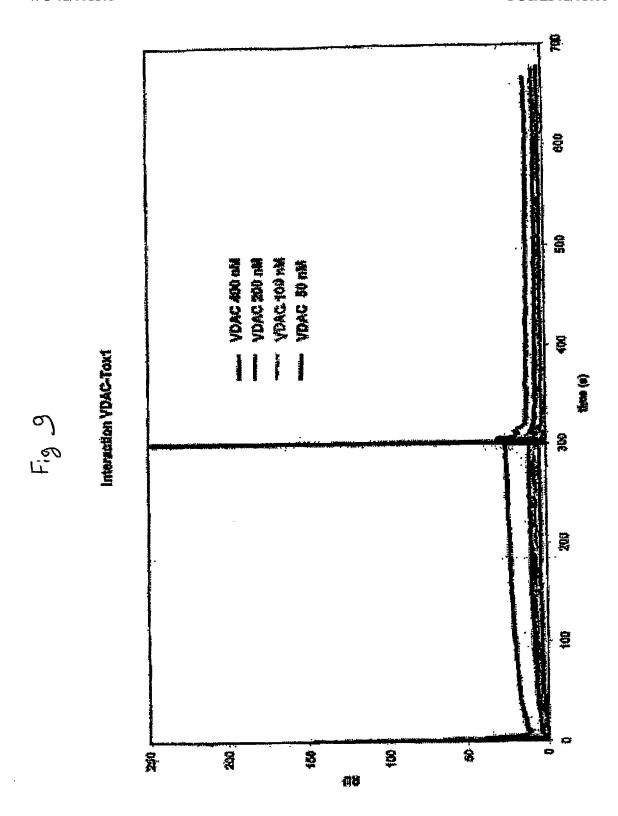
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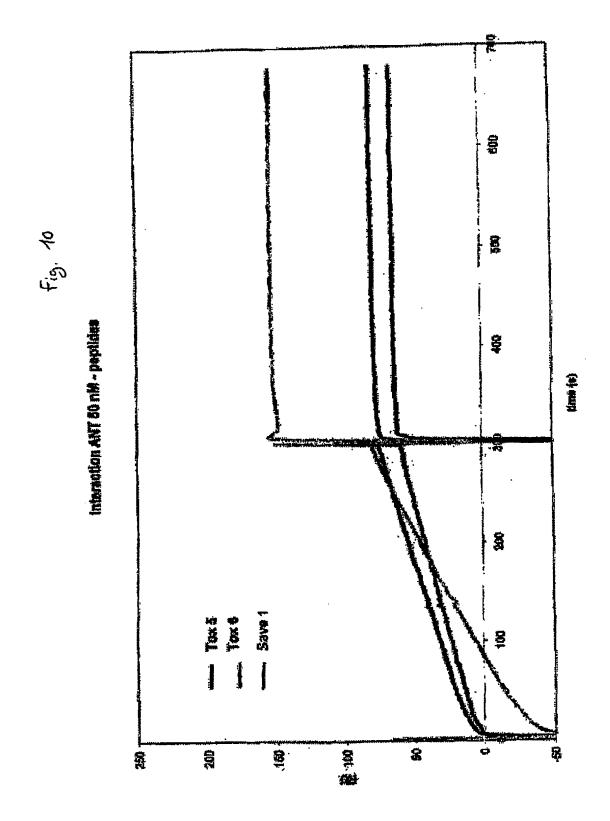
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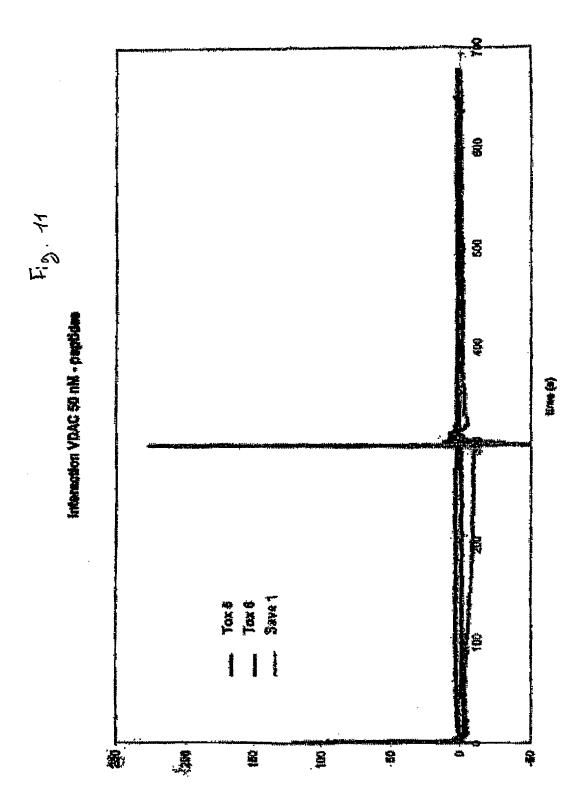












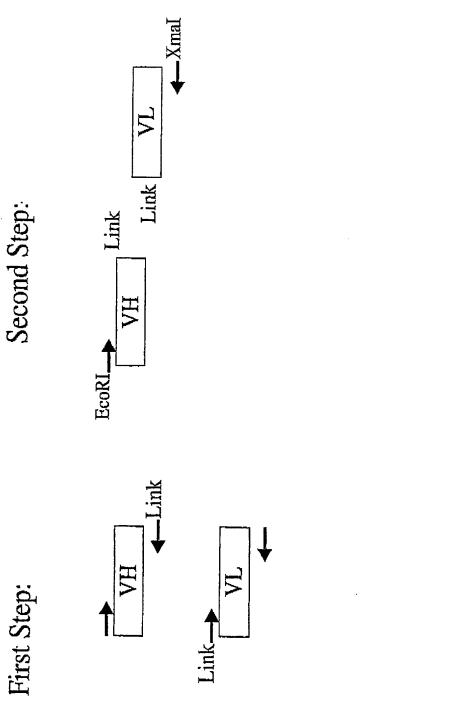


Figure 12: Obtention of the VH/VL chimeric DNA

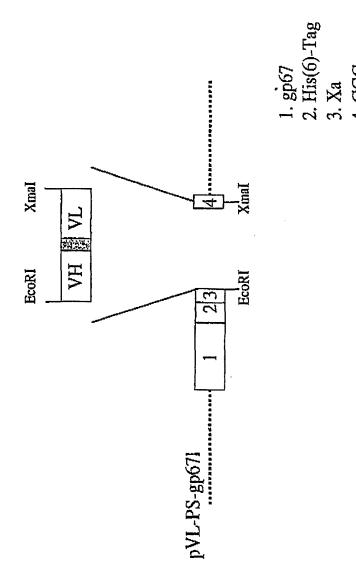


Figure 13: Map of the ScFv transfert vector